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THE RELATION OF KETO ACID EXCRETION TO AMINO ACID METABOLISM*

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(Received for publication, May 4, 1942)

Three keto acids other than acetoacetic acid have repeatedly been reported to be excreted in the urine under certain dietary or pathological conditions. The increase in the keto acid excretion in thiamine-deficient humans and rats is considered to be due to pyruvic acid (1). Phenylpyruvic acid is excreted in large amounts in cases of phenylpyruvic oligophrenia (2) and *p*-hydroxyphenylpyruvic acid has been found in the urine of premature infants (3). One case has been reported of tyrosinosis in which hydroxyphenylpyruvic acid was excreted in the urine (4).

After the administration of large amounts of tyrosine or phenylalanine to animals the corresponding keto acids are excreted (5).

Since most of the amino acids are deaminized to the corresponding keto acids during metabolism, a variety of keto acids is continuously generated in the organism. The fate of the naturally occurring carbonyl compounds is of particular interest in regard to abnormal nervous activity, since the ketonic grouping may be one of the configurations effective in suppressing convulsions induced experimentally (6). This suggestion is supported by the finding that pyruvic acid and acetoacetic acid suppress convulsions caused by the administration of thujone to rabbits (7). Such an effect of naturally occurring carbonyl compounds would be partly conditioned by their metabolic stability. Limited conclusions in this regard can be reached by feeding different amino acids to animals and determining the excretion of keto acids thereafter.

It is not known whether natural amino acids other than tyrosine or phenylalanine give rise to keto acid excretion under selected dietary conditions. Since normally a small amount of keto acids is excreted in human and animal urine, it is of interest to determine whether this fraction has its origin in the metabolism of amino acids.

In preliminary experiments the effect on keto acid excretion of the addition of different proteins to the protein-free diet was studied. It appears that the nature of the protein fed influences the extent of the subsequent keto acid excretion.

We have recently reported (8) that when rats are either fasted or placed on a protein-free, fat-carbohydrate diet there results an immediate de-

* This study was aided by a grant from the Joshua Rosett Research Fund.

crease of the keto acid excretion to a minimal level. On resumption of the administration of protein the keto acid excretion rapidly returns to its normal level.

When the minimal excretion level on the protein-free diet had been reached, different amino acids were added to the diet. The addition of glycine, *l*-glutamic acid, *l*-leucine, or *l*-alanine produced no increase in the keto acid excretion; *dl*-phenylalanine and *l*-tyrosine produced by far the greatest increase. *l*-Lysine and *dl*-methionine markedly increased the excretion, as did *dl*-isoleucine, *dl*-valine, *dl*-serine, *dl*-threonine, *dl*-aminobutyric acid, and *dl*-phenylaminobutyric acid. The 2,4-dinitrophenylhydrazones of the corresponding keto acid have been identified after the feeding of phenylalanine, methionine, tyrosine, valine, aminobutyric acid, phenylaminobutyric acid, and isoleucine.

EXPERIMENTAL

Urine Collection and Determination of Keto Acid Fraction—The rats were kept in glass cages on glass screens supported by large funnels. The feces were trapped by inverted watch-glasses in about the middle of the funnel. Glass wool was inserted in the funnel stem. Adapters led from the stem to large test-tubes (100 ml.) which were immersed in Dewar flasks containing solid carbon dioxide. This method of collecting the urine without the need of any preservative may be of use also for the determination of other labile substances.

Every 24 hours the funnels were rinsed with about 20 ml. of water, and the diluted urine was filtered. A modified Neuberg-Case procedure including the adaptations of Lu (9) and Harper and Deuel (10) was used for the determination of the keto acid fraction. For the color development 2 *N* sodium hydroxide was used according to Bueding and Wortis (11).

1 per cent of the diluted urine (or less when large amounts of keto acid were present) was pipetted into a 15 ml. centrifuge tube and treated with 1 ml. of saturated 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. The solution was then shaken three times with 2 ml. of ethyl acetate. The combined ethyl acetate layers were extracted with about 2 ml. of water and then three times with 2 ml. of 10 per cent sodium carbonate solution. To the combined carbonate extracts (6 ml.) were added 4 ml. of 2 *N* sodium hydroxide, and the extinction coefficient was measured in a photoelectric colorimeter with Filters 350 and 978 (Corning) combined.

Standard curves were prepared with the 2,4-dinitrophenylhydrazone of pyruvic acid which had been recrystallized three times from alcohol after it had attained a constant melting point (215.5° uncorrected). The urine values were read against this standard curve. Such a procedure appears to be justified, since with the filters selected the molar extinction coefficients

of the 2,4-dinitrophenylhydrazones of phenylpyruvic acid, hydroxyphenylpyruvic acid, α -keto- γ -methiobutyric acid, α -ketobutyric acid, acetoacetic acid, oxalacetic acid, and α -keto- β -methylvaleric acid agreed within ± 10 per cent with that of the corresponding derivative of pyruvic acid.

In order to test the accuracy of the procedure, solutions of redistilled pyruvic acid were treated the same way as urine. The recovery of samples containing 14 to 55 γ per ml. averaged 96 ± 5 per cent. Urine without addition of the hydrazine gives a blank value, whereas the hydrazine solution gives color values corresponding to 1 to 2 γ of pyruvic acid, equivalent to 0.1 to 0.2 mg. of pyruvic acid for the final urine values.

The color developed by the mixed hydrazones extracted from urine fades slowly after addition of the 2 N sodium hydroxide to the carbonate solution. It was hoped that the rate of fading might be indicative of one prevalent hydrazone. The fading curves of the hydrazones of five different keto acids were therefore determined in five different concentrations ranging from 20 γ to 100 γ per ml. After 90 minutes at room temperature the extinction coefficient of the hydrazone solution from pyruvic acid, ketoglutaric acid, phenylpyruvic acid, α -keto- γ -methiobutyric acid, and acetoacetic acid corresponded to 95, 96, 66, 88, and 91 per cent respectively of the values obtained immediately after the carbonate solution was mixed with 2 N NaOH. For the different hydrazone concentrations of the same keto acid, the fading curves were parallel with the exception of that of the hydrazone of phenylpyruvic acid. This faded at a faster rate at lower concentrations. The hydrazones of the mixed keto acids from the urine of twelve rats on a normal rat ration were tested for fading. After 90 minutes, 83 to 86 per cent of the original values was found, indicating that a major portion of the keto acid fraction of normal rat urine does not correspond to keto acids of which the hydrazones fade relatively slowly.

Metabolic Experiments—Two colonies of inbred male Wistar rats kept on stock Rockland rat diet were used for the experiments. The rats of one colony had somewhat higher fasting values than those of the other.

The following experiments describe the effect of short fasting and of a protein-free diet on the urinary keto acid output of rats. Four male rats (270 to 340 gm.) were kept on the stock diet for 8 days. Their keto acid excretion ranged from 1 to 4 mg. daily. Food was withheld and on the 1st day of fasting the output dropped to 0.7 to 1.1 mg., on the 2nd to 0.4 to 0.6 mg., and on the 3rd to 0.3 to 0.5 mg. The food was returned to the cages, and on the next day the excretion values ranged from 0.9 to 1.5 mg., and increased to 1.0 to 4.5 mg. the day after. After 5 days of stock diet and 1 day of fasting the animals were put on the protein-free diet (30 per cent lard, 40 per cent sucrose, 25 per cent dextrin, 5 per cent salt mixture (12)). The next day the keto acid excretion was found to be 0.6 to 0.8 mg.

The amino acids¹ were fed to rats in amounts of 0.01 mole for 10 gm. of protein-free diet daily for 2 days after the keto acid excretion had been brought down to its minimal level. Lysine, arginine, histidine, and cys-

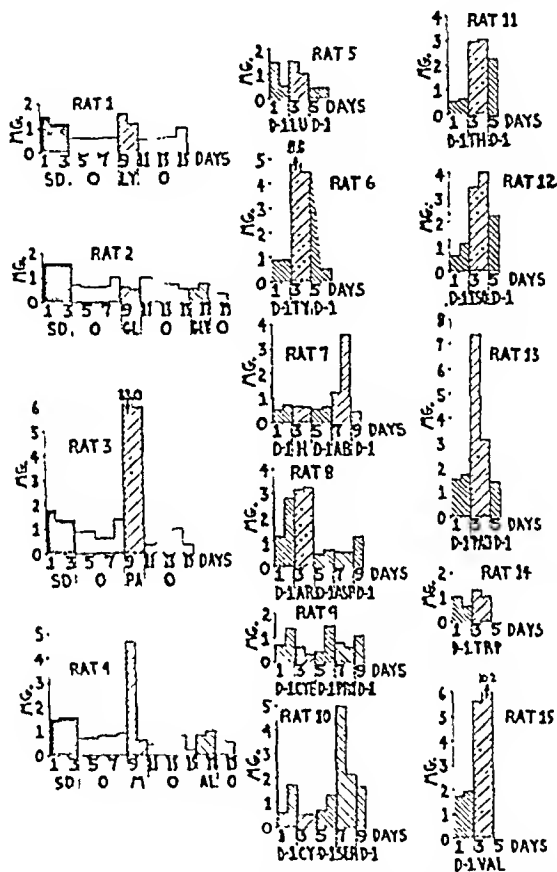


FIG. 1. Daily urinary excretion of keto acids after amino acids were fed to rats (expressed in mg. of pyruvic acid). SD = stock Rockland rat diet; O = fasting; D-1 = Diet I (lard 30, sucrose 40, dextrin 25, salt mixture 5 (12)); LY = lysine; GL = glutamic acid; GLY = glycine; PA = phenylalanine; M = methionine; AL = alanine; LU = leucine; TY = tyrosine; H = histidine; AB = aminobutyric acid; AR = arginine; ASP = aspartic acid; CYE = cysteine; PRO = proline; CY = cystine; SER = serine; TH = threonine; ISO = isoleucine; PAB = phenylaminobutyric acid; TRP = tryptophane; VAL = valine.

teine were added to the diet as their hydrochlorides, with an equivalent amount of sodium bicarbonate. The experiments reported in Fig. 1 are

¹ Some of the amino acids were obtained from the University of Illinois and some from Merck and Company, Inc. For the *dl*-isoleucine and the *dl*-phenylaminobutyric acid we are indebted to Dr. du Vigneaud.

representatives of two or more which were carried out the same way. In most of the experiments 50 per cent or more of the food was consumed. The rats were weighed every day, and in most of the experiments the daily nitrogen output was determined. These data are not reported, since they seem to be of no particular bearing at this stage of the investigation.

The largest increase in keto acid output was found after the feeding of *l*-tyrosine and *dl*-phenylalanine, less with the other five racemic amino acids. The hydrazones of the seven keto acids were isolated and identified. Increased excretion was also found in the experiments with *l*-lysine, *dl*-serine, and *dl*-threonine but we were unable to identify the hydrazones. Leucine and arginine, in some experiments, gave an increase which was within the limits of individual fluctuations and therefore not significant. In several experiments the keto acid excretion was higher on the 1st day of amino acid feeding, since the rats consumed more food on the 1st than on the 2nd day.

Isolation and Properties of 2,4-Dinitrophenylhydrazones—To the filtered urines, diluted as little as possible, was added an excess of 2,4-dinitrophenylhydrazine in hydrochloric acid. After 16 hours in the ice box, the hydrazones were centrifuged off and recrystallized several times from alcohol-water, alcohol, and ethyl acetate. In experiments with increased keto acid output the addition of the hydrazine solution resulted in an immediate cloudiness.

Hydrazone of α -keto β methylbutyric acid, m p 194° (13) Found, C 44.6, H 4.5, calculated, C 44.59, H 4.03

Hydrazone of α -keto- γ -methiobutyric acid, m p 149° (14) Found, S 9.57, calculated, S 9.77

Hydrazone of α -ketobutyric acid, m p 195° (13) Found, C 42.90, H 3.90; calculated, C 42.56, H 3.57

Hydrazone of γ phenyl- α ketobutyric acid, m p 168° Found, C 53.63, H 3.95; calculated, C 53.64, H 3.94

Hydrazone of hydroxyphenylpyruvic acid, m p 177° (sintered at about 156°). Found, C 49.64, H 3.33, calculated, C 49.99, H 3.36

Hydrazone of α keto β methylvaleric acid, m p. 170° Found, C 46.74, H 4.5, calculated, C 46.46, H 4.55

Hydrazone of phenylpyruvic acid, m p 189° Mixed m p with synthetic sample 189°. This hydrazone was the most difficult to isolate and to purify, possibly due to the presence of the corresponding derivative of hydroxyphenylpyruvic acid.

The dinitrophenylhydrazones of α -ketobutyric acid, γ -phenyl- α -ketobutyric acid, and hydroxyphenylpyruvic acid,² like that of α -keto- γ -

² The different forms may account for different melting points found for the hydrazone of hydroxyphenylpyruvic acid and ketomethiobutyric acid. Krebs and Felix and Zorn (13) have found 158° for the former and Duschinsky and Jeannerat (15) 128° for the latter. Dr R. Duschinsky has kindly informed us that other preparations of the hydrazone of α -keto- γ methiobutyric acid melted at 149° as ours did.

methiobutyric acid (16), occurred in two forms different in color and solubility.

Rôle of Proteins in Diet and Keto Acid Excretion—Since the stock diet which contains plant proteins³ is not as well defined as a synthetic diet with known protein components, the experiments have been repeated with diets

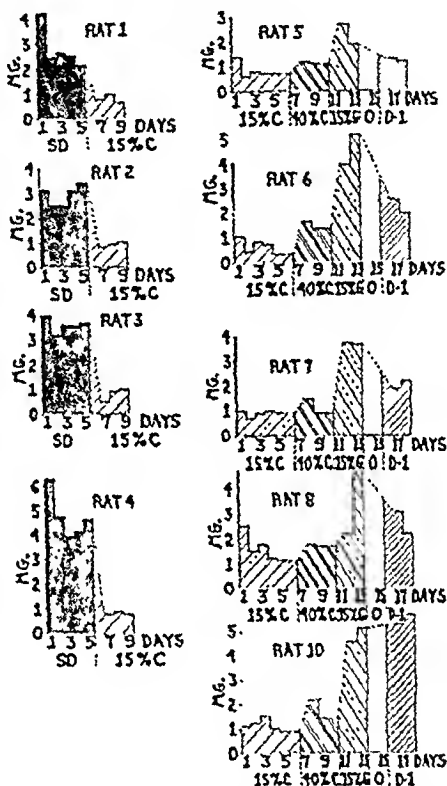


FIG. 2. Daily urinary excretion of keto acids after protein-containing diets were fed to rats (expressed in mg. of pyruvic acid). SD = Rockland rat diet; 15% C = casein (Labro, vitamin-free) 15, lard 24, sucrose 30, dextrin 20, salt mixture 5, cod liver oil 5, ryzamin-B₂ 1; 40% C = casein 40, lard 24, sucrose 20, dextrin 10, salt mixture 5; 15% G = gelatin 15 in place of casein in 15% C; D-1 = Diet I, see Fig. 1; 0 = fasting.

containing 15 per cent casein, blood albumin, or gelatin, or 40 per cent casein (Fig. 2). Contrary to expectation, the feeding of the diet containing 15 per cent casein resulted in a decrease of the keto acid excretion to the

³ Menthol glucuronic acid and glucuronic acid, for which we are indebted to Dr. Huebner, as well as ascorbic acid were negative when tested in the colorimetric procedure.

level of the protein-free diet. This was true regardless of whether the diet of the rats was restricted to 10 gm. per day or not, or whether the vitamin B complex was included in the diet or not.

Rats 5 to 7 (Fig. 2) weighed 214 to 222 gm. at the beginning of the experiment, Rats 8 and 10, 500 and 423 gm. It seems that the older rats had a somewhat higher keto acid excretion on the 15 per cent casein diet than did those which were still growing. The feeding of the 15 per cent gelatin diet resulted in a definite increase in the keto acid output, reaching the level of that with the stock diet. The younger rats seem to return to the lower level faster than the older ones after having been put on the protein-free diet. Diets containing 15 per cent of blood albumin resulted in an increase of the keto acid excretion similar to that induced during feeding of the gelatin-containing diet.

DISCUSSION

Only two of the twelve amino acids fed as the natural isomers, namely tyrosine and lysine, induced a definite increase in keto acid excretion, whereas all nine amino acids fed as the racemic compounds did so. It may well be that the increase in keto acid output from *dl*-amino acids was due to the presence of the unnatural isomer, which when deaminized in the kidney gives rise to a high concentration of keto acid in this organ. Further study will be necessary to show whether this is the case, and whether the keto acids excreted after feeding serine, threonine, and lysine are related to the amino acid fed. This would be of particular interest because of the unknown metabolic pathway of these compounds. No attempt has been made to learn anything about the fate of the portion of the amino acid not converted into the keto acid. It may be expected that some of the amino acids give rise to the excretion of the hydroxy derivatives (5), of the acetylated amino acid (17), and of the unchanged unnatural isomer (18). With the above exceptions, the excreted keto acids were identified as corresponding to the amino acids fed. The amount of keto acids excreted ranged in the case of phenylalanine from 4 to 7 per cent, tyrosine from 1.1 to 6 per cent, and in the case of the other amino acids from 0.1 to 1.0 per cent of the amino acid consumed.

The excretion of the keto acids in the urine after the feeding of the natural isomers of phenylalanine (5), tyrosine, and lysine indicates the possibility that certain amino acids may also contribute to the keto acid fraction of the urine when taken in as protein. The direct proof lies in the identification of the keto acids after protein feeding. Not only the presence but the nature of the protein fed appears to be of decisive influence on the keto acid output. Casein keeps the keto acid excretion on the level of that of fasting rats or of rats fed a protein-free diet. The decrease in keto acid

excretion during short fasts in normal and thiamine-deficient animals has been observed by Harper and Deuel (10) and by Shils and colleagues (19). Rats fed a stock diet, or diets containing blood albumin or gelatin as the protein component, have a high urinary keto acid output. These results suggest that proteins which are readily utilized and well balanced in their amino acid composition may not produce an increased keto acid output. However, it is possible that the keto acids which appear after the feeding of certain proteins are not derived from the protein itself but are the results of specific stimulation of other metabolic processes.

The arrest of growth in growing animals may result in an increased excretion of keto acids from proteins only partially utilized for growth purposes. Whether such a mechanism would contribute to the increase in pyruvic acid output by thiamine-deficient animals remains to be decided by actual isolation of the excreted keto acids.

SUMMARY

The urinary excretion of keto acids was studied in rats during fasting, on a protein-free diet, and after single amino acids and proteins were fed. The keto acids were determined with a modified Neuberg-Case procedure. The values obtained can be expressed in pyruvic acid equivalents, since the molar extinction coefficient of the 2,4-dinitrophenylhydrazones of seven naturally occurring keto acids agrees within ± 10 per cent of that of the hydrazone of pyruvic acid. The colors developed by various hydrazones in alkaline solutions fade slowly and at different rates. The fading curves of the hydrazones of the keto acids from rat urine indicate the presence of a mixture of keto acids. Rats on the stock diet excrete from 1 to 6 mg. of keto acids (expressed as pyruvic acid) daily. In fasting rats or in rats on a protein-free diet, the excretion drops immediately to values of 1 mg. or less.

Rats kept on the protein-free diet until the minimal excretion level was reached received one of twenty-one different amino acids daily for 2 days in addition to the diet. A definite increase in keto acid excretion occurred following the feeding of two of twelve *L*-amino acids, namely tyrosine and lysine, and following the feeding of *DL*-phenylalanine, *DL*-valine, *DL*-methionine, *DL*-aminobutyric acid, *DL*-phenylaminobutyric acid, *DL*-isoleucine, *DL*-threonine, and *DL*-serine. The hydrazones of the keto acids corresponding to the first six *DL*-amino acids have been isolated from the urine and characterized. In the case of tyrosine and phenylalanine only the excretion of the keto acids amounted to more than 1 per cent of the amino acid consumed.

Feeding a diet containing 15 per cent of casein results in a drop of keto acid excretion to the fasting level. Addition of gelatin or blood albumin to the diet results in an increase in the keto acid excretion to the level obtained with rats on the stock diet.

BIBLIOGRAPHY

1. Platt, B. S., and Lu, G. D., *Quart. J. Med.*, **5**, 355 (1936).
2. Foelling, A., *Z. physiol. Chem.*, **227**, 169 (1934).
3. Levine, S. Z., Marples, E., and Gordon, H. H., *J. Clin. Inv.*, **20**, 199 (1941).
4. Medes, G., *Biochem. J.*, **26**, 217 (1932).
5. Kotake, Y., Matsuoka, Z., and Okagawa, M., *Z. physiol. Chem.*, **122**, 166 (1922).
Kotake, Y., Masai, Y., and Mori, Y., *Z. physiol. Chem.*, **122**, 195 (1922).
Shambaugh, N. F., Lewis, H. B., and Tourtelotte, D., *J. Biol. Chem.*, **92**, 499 (1931).
Foelling, A., Closs, K., and Gamnes, T., *Z. physiol. Chem.*, **256**, 1 (1938).
Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, **135**, 251 (1940).
6. Putnam, T. J., and Merritt, H. H., *Arch. Neurol. and Psychiat.*, **45**, 505 (1941).
7. Keith, H. M., *Arch. Neurol. and Psychiat.*, **29**, 148 (1933).
8. Waelsch, H., *J. Biol. Chem.*, **140**, 313 (1941).
9. Lu, G. D., *Biochem. J.*, **33**, 249 (1939).
10. Harper, H. A., and Deuel, H. J., Jr., *J. Biol. Chem.*, **137**, 233 (1941).
11. Bueding, E., and Wortis, H., *J. Biol. Chem.*, **133**, 585 (1940).
12. Hawk, P. B., and Oser, B. L., *Science*, **74**, 369 (1931).
13. Krebs, H. A., *Z. physiol. Chem.*, **217**, 220 (1933).
Felix, K., and Zorn, K., *Z. physiol. Chem.*, **247**, 141 (1937); **258**, 16 (1939).
14. Waelsch, H., and Borek, E., *J. Am. Chem. Soc.*, **61**, 2252 (1939).
15. Duschinsky, R., and Jeannerat, J., *Compt. rend. Acad.*, **208**, 1359 (1939).
16. Borek, E., and Waelsch, H., *J. Biol. Chem.*, **141**, 99 (1941).
17. Knoop, F., and Kertess, E., *Z. physiol. Chem.*, **71**, 252 (1911).
du Vigneaud, V., and Irish, O. J., *J. Biol. Chem.*, **122**, 349 (1937-38).
18. Wohlgemuth, J., *Ber. chem. Ges.*, **38**, 2069 (1915).
19. Shils, M. E., Day, H. G., and McCollum, E. V., *J. Biol. Chem.*, **139**, 145 (1941).

THE PHYSICAL AND CHEMICAL PROPERTIES OF A DISTINCTIVE STRAIN OF TOBACCO MOSAIC VIRUS

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It has been apparent for some time in both plant and animal virus diseases that the extent and type of the disease produced in a particular host may vary widely according to the strain of virus involved, and that members of the same virus family may range in pathogenicity from a strain which is quite innocuous to one which is definitely lethal. These facts make it of unusual interest to elucidate the nature of the chemical differences in the structure of a virus which are responsible for such variations in the biological properties. The nucleoproteins of the tobacco mosaic virus family are particularly suitable for such a study, for in many cases they can be obtained in comparatively large amounts and in a degree of purity superior to that of most other plant and animal viruses.

At this stage in the investigation of chemical differences between strains, the variants which differ most widely from the type strain with respect to biological properties seem to offer the best opportunity for demonstrating structural differences. Recently, Dr. Holmes isolated from rib-grass (*Plantago lanceolata* L.) a strain of tobacco mosaic virus which possesses unique properties and which can be easily distinguished from previously known strains by its ability to form necrotic ring lesions in Turkish tobacco, by its adaptation to rib-grass, and by its failure to produce local lesions in bean plants (*Phaseolus vulgaris* L.) (1). This unusual variant was shown beyond reasonable doubt to be a strain of tobacco mosaic virus by its ability to withstand heat and desiccation, by its inability to infect tobacco plants diseased with typical tobacco mosaic virus, by its precipitation with tobacco mosaic virus antiserum, and by the nature of its response to the genic constitution of tobacco plants. The present communication deals with the isolation and purification of the new virus and with the examination of some of its physical, chemical, and serological properties.

Preparation of the Virus—Young Turkish tobacco plants were inoculated with the rib-grass virus by rubbing one or two leaves on each plant with a gauze pad saturated with infective juice. This juice was obtained from a tobacco plant showing the typical symptoms of disease described by Holmes (1). About a month after inoculation, the plants were harvested and frozen. The virus was subsequently extracted from the macerated plant

tissue, filtered through celite, and purified by differential centrifugation. The isolation and purification procedures corresponded in essential details with those commonly employed in this laboratory and recently used in the preparation of cucumber virus 4 (2).

The yields of purified virus obtained from diseased Turkish tobacco, while definitely smaller than those customarily obtained with ordinary tobacco mosaic virus, were nevertheless good. From 0.4 to 0.8 gm. of highly purified virus was obtained per liter of expressed juice as compared with 2 to 2.5 gm. per liter of the type strain obtained under similar conditions.

The rib-grass virus was tested for specific activity on *Nicotiana glutinosa* L. by the half leaf method. Various dilutions of the highly purified virus in 0.1 M phosphate buffer were compared with an arbitrary standard containing 10^{-4} gm. of virus per ml. The number of lesions obtained under these conditions was roughly proportional to the virus concentration in the range 10^{-4} to 10^{-6} gm. per ml. Concentrations below and above the latter range yielded fewer and more lesions, respectively, than would be expected from the virus concentration. This result is commonly obtained with other strains of tobacco mosaic virus. An almost identical number of lesions was obtained when the rib-grass virus was compared with ordinary tobacco mosaic virus on *Nicotiana glutinosa* at concentrations of 10^{-4} gm. per ml. However, as noted by Holmes (1), the lesions produced by the rib-grass strain were considerably smaller than those produced by ordinary tobacco mosaic virus and were also slightly different in character. The maximum infective dilution of the rib-grass virus appeared to be in the neighborhood of 10^{-10} gm. per ml. This value is comparable to that obtained with the ordinary strain.

Crystallization of the Virus—The distinctive properties of the rib-grass strain made it seem desirable to obtain the virus in crystalline form for the purpose of comparing it with ordinary tobacco mosaic virus. Crystallization was accomplished by adding slowly, with stirring, a 1:20 mixture of glacial acetic acid and 0.5 saturated ammonium sulfate to a 0.6 per cent solution of highly purified virus to which enough ammonium sulfate had been added to cause a faint turbidity (3). When the solution became very turbid, a few drops of saturated ammonium sulfate were added with vigorous stirring. Crystallization occurred almost immediately, to yield needles which appeared to be of the same type as the paracrystals of ordinary tobacco mosaic virus.

Elementary and Carbohydrate Analyses—Virus preparations obtained by differential centrifugation were further purified by electrodialysis in the type of cell described by Albanese (4). Dialysis was discontinued when the current dropped to a constant level. The drop in current was almost

always accompanied by a partial or complete precipitation of the salt-free virus. A suspension of the virus was then frozen and dried *in vacuo*. The white fluffy material thus obtained was further dried to constant weight at 110° in a drying oven and used for analyses. That little or no disintegration of the virus occurred during electrodialysis was demonstrated by the fact that virus precipitated by electrodialysis, when redissolved in dilute phosphate buffer, produced fully as many lesions on *Nicotiana glutinosa* as equivalent amounts of the highly active undialyzed virus.

Most of the analyses, with the exception of those for carbohydrate and phosphorus, were made by Dr. A. Elek, of this Institute, using customary micromethods. Carbohydrate and phosphorus were determined by standard colorimetric procedures as described recently for cucumber virus 4 (2, 5, 6). The average of duplicate analyses on five preparations of the virus indicated the presence of 50.28 per cent carbon, 6.98 per cent hydrogen, 15.69 per cent nitrogen, 0.64 per cent sulfur, 0.54 per cent phosphorus, 2.27 per cent ash, and 2.35 per cent carbohydrate. These values, with the exception of that for sulfur, agree fairly well with analyses on ultracentrifugally prepared tobacco mosaic virus of the ordinary type. However, the rib-grass strain appears to contain about 3 times as much sulfur as the type strain. The nature of this comparatively large amount of sulfur is at present under investigation. Preliminary tests have indicated that at least part of the sulfur is probably present as $-SH$, since even mildly denatured virus gave a strongly positive nitroprusside test.

Nucleic Acid—Nucleic acid was readily separated from the protein part of the virus by treatment with alkali, as described by Johnson and Harkins (7). The protein-free nucleic acid, obtained in the form of a dry white powder, was found to contain 34.10 per cent carbon, 3.93 per cent hydrogen, 15.80 per cent nitrogen, and 9.00 per cent phosphorus. It gave a strongly positive test for pentose with orcinol in hydrochloric acid, and a negative test for desoxypentose with diphenylamine in acetic acid. From these tests it may be concluded that the nucleic acid of the rib-grass strain, like that of ordinary tobacco mosaic virus, is of the ribonucleic acid or yeast type.

Size and Shape of the Virus—Solutions of the rib-grass virus were found to exhibit anisotropy of flow. Moreover, when a solution of the virus was caused to flow, the entire stream was doubly refracting and the double refraction persisted for a time after the stream left a pipette. These facts indicated that the rib-grass virus possessed the peculiar rod shape which is so characteristic of tobacco mosaic virus and its strains. It was, therefore, of considerable interest to compare the average size of these rods with that of ordinary tobacco mosaic virus.

Preparations of the virus were examined in the analytical ultracentrifuge by Dr. Max A. Lauffer. At dilutions of 1.0, 2.1, and 3.5 mg. of virus per ml., sedimentation constants of 187×10^{-13} , 187×10^{-13} , and 183×10^{-13} , respectively, were obtained. These values correspond closely to those obtained for ordinary tobacco mosaic virus at corresponding dilutions (8).

Electron micrographs of the virus were made by Dr. T. F. Anderson, RCA Fellow of the National Research Council, using an electron microscope made available by the Radio Corporation of America, Camden, New Jersey. Like other strains of tobacco mosaic virus, the particles observed in micrographs of the rib-grass strain showed a considerable variation in size. A comparison of micrographs of ordinary tobacco mosaic virus and the rib-grass strain revealed no obvious difference in the average size of the particles. X-ray measurements have indicated an average diameter of about $15 \text{ m}\mu$ for particles of the rib-grass virus (1), which is the same value obtained for three other strains of tobacco mosaic virus, including the ordinary form (9).

It may be concluded from the combined sedimentation, electron microscope, and x-ray data that the particles of tobacco mosaic virus and the rib-grass strain possess no readily demonstrable differences in size and shape. A particle size of about $15 \text{ m}\mu$ in width and $280 \text{ m}\mu$ in length and a molecular weight of about 4×10^7 have been assigned to ordinary tobacco mosaic virus (10, 11).

Ultraviolet Absorption Spectra—In order to characterize further the rib-grass virus, its ultraviolet absorption was compared with that of ordinary tobacco mosaic virus. Preparations of the intact viruses, their protein components, and their nucleic acids were examined by Dr. G. I. Lavin, using the techniques described in previous publications (12, 13). The protein components were those used in other experiments (14), and the nucleic acids were prepared by the method of Johnson and Harkins (7). The absorption coefficient, α , was calculated in each case from the concentration of material expressed as mg. per ml. The absorptions of the two viruses and of their protein components were similar and those of their nucleic acid components were almost indistinguishable. The intact viruses showed a maximum absorption at about 2675 \AA . and the proteins showed a maximum absorption at about 2800 \AA . The relative positions of maxima and minima and the shapes of the curves are those to be expected from nucleoproteins and simple proteins, respectively (13). The absorption maxima of the nucleic acid components of the two viruses were at about 2600 \AA ., which is comparable to the values obtained for a number of other nucleic acids from various sources (13, 15, 16). While there appeared to be little difference between the absorption curves for tobacco mosaic and

the rib-grass viruses, and of their protein components, there were definite differences in the band structures as obtained with the continuous light of the hydrogen discharge tube. These differences were most pronounced in the tyrosine-tryptophane region, and in this respect are in accord with data dealing with the chemical composition of the two viruses (17).

Serological Tests—Precipitin tests with six strains of tobacco mosaic virus and cucumber viruses 3 and 4 as antigens and tobacco mosaic virus antiserum demonstrated a strong serological relationship between tobacco mosaic virus and all of the viruses tested, with the exception of the rib-grass strain and cucumber viruses 3 and 4 (17). A comparatively weak relationship was observed between the latter viruses and ordinary tobacco mosaic virus. As a corollary to these experiments, tests were made in the present study with antiserum to the rib-grass virus. This antiserum was obtained from the blood of a rabbit 8 to 10 days after the last of five spaced intravenous injections of a total of about 40 mg. of virus. Precipitin tests were made with a constant dilution of serum and with various dilutions of antigen according to the technique described in a previous communication (2). All of the viruses used as antigens were highly purified preparations obtained by differential centrifugation. Instead of the expected weak precipitation of strains against the rib-grass virus antiserum, precipitates were obtained which were qualitatively indistinguishable from those of the rib-grass virus itself. Subsequent repetition of the tests with sera obtained from two other rabbits yielded the same results.

Experiments were performed next in which separate portions of tobacco mosaic virus antiserum were absorbed with tobacco mosaic and rib-grass viruses. Portions of antiserum to the rib-grass virus were treated in a similar fashion. The total precipitate obtained in each absorption was suspended in water and analyzed for nitrogen by the Kjeldahl method. The results of the latter analyses indicated that about 0.6 as much precipitate was obtained when the rib-grass virus reacted with tobacco mosaic virus antiserum as when tobacco mosaic virus reacted with an equal portion of the same antiserum. On the other hand, more than 0.8 as much precipitate was obtained when tobacco mosaic virus reacted with rib-grass virus antiserum as was obtained in the homologous reaction. Thus, the quantitative reactions confirmed the qualitative tests which had shown unequal cross-precipitation of the two strains with the appropriate antisera.

Precipitin tests were also made with absorbed sera and strains of tobacco mosaic virus, as shown in Table I. The dilutions and other conditions of these tests were exactly the same as used for previous precipitin reactions with unabsorbed sera. The results obtained agreed with and extended

those reported by Chester (18) and by Bawden and Pirie (19) in showing that strains of tobacco mosaic virus possess both distinctive and common antigens.

In general, the serological reactions observed indicate that considerable information regarding strains of tobacco mosaic virus, and possibly the nature of antigen-antibody reactions as well, could be obtained by quantitative serological studies, perhaps of the Heidelberger type (20), par-

TABLE I

Precipitation of Strains of Tobacco Mosaic Virus with Absorbed Antisera

The signs indicate the degree of precipitation.

Virus	Dilution of antigen (1.1 = 1 mg per ml)			
	1:1	1:4	1:16	1:64
Tobacco mosaic virus antiserum absorbed with rib-grass virus				
Holmes' rib-grass	-	-	-	-
Tobacco mosaic	++	+++	++	+
Yellow aucuba	-	-	+	+
Green "	-	+	+	+
Holmes' masked	+	+++	++	+
J14D1	++	++	+	+
Cucumber virus 4	-	-	±	-
Rib-grass virus antiserum absorbed with tobacco mosaic virus				
Holmes' rib-grass	+++	++	+	+
Tobacco mosaic	-	-	-	-
Yellow aucuba	++	+	-	-
Green "	+	±	-	-
Holmes' masked	-	-	-	-
J14D1	-	±	-	-
Cucumber virus 4	+++	++	+	+

ticularly if such data were correlated with evidence now being obtained regarding the chemical composition of these unusual antigens.

DISCUSSION

The general composition, physicochemical properties, and size and shape of the rib-grass virus were found to agree in almost every respect with the similar properties of ordinary tobacco mosaic virus. These results may not, at first, appear surprising, because the rib-grass virus completely satisfies every important criterion for classification as a strain of tobacco mosaic virus, and hence would be expected to resemble closely the type strain. However, when it is recalled that the rib-grass virus has

been found to contain about 3 times as much sulfur as the type strain and to differ strikingly from the latter virus in content of aromatic amino acids (17), the coincidence of properties of the two viruses becomes remarkable.

The author wishes to express his appreciation to Dr. F. O. Holmes for the original supply of the rib-grass virus and to Dr. W. M. Stanley for encouragement and helpful suggestions during the course of this investigation.

SUMMARY

A distinctive strain of tobacco mosaic virus, originally discovered in rib-grass, has been isolated from diseased Turkish tobacco and obtained in a highly purified state by differential centrifugation. The new virus, like ordinary tobacco mosaic virus, could be obtained in the form of needle-like paracrystals. Elementary and carbohydrate analyses gave values similar to those obtained with ultracentrifugally prepared tobacco mosaic virus with one exception. The rib-grass virus contained about 0.64 per cent sulfur in comparison to only 0.2 per cent sulfur reported for ordinary tobacco mosaic virus. Nucleic acid was separated from the protein component of the virus and found to be of the ribonucleic acid type.

Solutions of the virus were doubly refracting and examination in the analytical ultracentrifuge and in the electron microscope indicated the presence of rod-like particles which in size and shape appeared indistinguishable from those of common tobacco mosaic virus.

Ultraviolet absorption curves were obtained for the rib-grass and tobacco mosaic viruses, their protein components, and their nucleic acids. There appeared to be little difference between the absorption curves of tobacco mosaic and the rib-grass viruses and of their protein and nucleic acid components. However, a significant difference between the two viruses was apparent in the band structure as obtained with the continuous light of the hydrogen discharge tube, particularly in the tyrosine-tryptophane region. This result is in accordance with the chemical data previously reported from this laboratory.

Serological tests indicated that the rib-grass virus and ordinary tobacco mosaic virus contain common antigenic groups but, in addition, that each possesses distinctive groups lacking in the other.

BIBLIOGRAPHY

1. Holmes, F. O., *Phytopathology*, **31**, 1089 (1941).
2. Knight, C. A., and Stanley, W. M., *J. Biol. Chem.*, **141**, 29 (1941).
3. Stanley, W. M., *Science*, **81**, 644 (1935).
4. Albanese, A. A., *J. Biol. Chem.*, **134**, 467 (1940).

5. Tillmans, J., and Philippi, K., *Biochem. Z.*, **215**, 36 (1929).
6. King, E. J., *Biochem. J.*, **26**, 292 (1932).
7. Johnson, T. B., and Harkins, H. H., *J. Am. Chem. Soc.*, **51**, 1779 (1929).
8. Lauffer, M. A., *J. Physic. Chem.*, **44**, 1137 (1940).
9. Bernal, J. D., and Fankuchen, I., *J. Gen. Physiol.*, **25**, 111 (1941).
10. Stanley, W. M., and Anderson, T. F., *J. Biol. Chem.*, **139**, 325 (1941).
11. Lauffer, M. A., *Science*, **87**, 469 (1938).
12. Lavin, G. I., and Stanley, W. M., *J. Biol. Chem.*, **118**, 269 (1937).
13. Lavin, G. I., Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, **130**, 259 (1939).
14. Knight, C. A., and Lauffer, M. A., *J. Biol. Chem.*, **144**, 411 (1942).
15. Lavin, G. I., Thompson, R. H. S., and Dubos, R. J., *J. Biol. Chem.*, **125**, 75 (1938).
16. Hoagland, C. L., Lavin, G. I., Smadel, J. E., and Rivers, T. M., *J. Exp. Med.* **72**, 139 (1940).
17. Knight, C. A., and Stanley, W. M., *J. Biol. Chem.*, **141**, 39 (1941).
18. Chester, K. S., *Phytopathology*, **26**, 778 (1936).
19. Bawden, F. C., and Pirie, N. W., *Brit. J. Exp. Path.*, **18**, 275 (1937).
20. Heidelberger, M., *Bact. Rev.*, **3**, 49 (1939).

CARBON SUBOXIDE AND PROTEINS

VI. CHYMOTRYPTIC DIGESTION OF MALONYL EGG AND SERUM ALBUMINS*

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Earlier studies from this laboratory (1) have shown that the reaction of carbon suboxide, C_3O_2 , with proteins results in the introduction of malonyl residues on free amino and phenolic hydroxyl groups. The reagent behaves like ketene, except that the acyl group, which it forms, cancels the positive charge of amino groups ($+H_3N \cdot R$), at the same time contributing a negative charge of its own ($-OOC \cdot CH_2 \cdot CO \cdot NH \cdot R$). The original plan of studying the effect of this change upon the specificity of proteolytic enzymes is now under way; meanwhile we have investigated the hydrolysis by chymotrypsin of two native and two malonylated albumins, hoping that the knowledge thus gained might aid in the clarification of the action of this enzyme.

The properties of the derived proteins employed are given by the data in Table I. The horse serum albumin was the crystalline carbohydrate-free protein prepared by McMeekin's method (2). The egg albumin was crystallized according to the method of Kekwick and Cannan (3), and the chymotrypsin according to that of Kunitz and Northrop (4). The experimental details for adding carbon suboxide to proteins and the analytical procedures developed for its use have already been described (1).

It was first determined that malonylation of the native protein has no important effect upon the pH of optimum hydrolysis by chymotrypsin. The data, plotted in Fig. 1, indicate that the optimum for carbon suboxide-treated serum albumin may lie 0.5 pH unit below that for the native protein; this difference was respected in the experimental work, although it is not considered very significant. These preliminary experiments did, however, indicate that malonylated egg albumin is much more readily digested during the initial stages than is the native protein.

This was also observed when exhaustive studies of the digestion were carried out (Fig. 2). During the first few hours the rate of digestion of the malonylated protein was several times that of the native protein, but after the initial stages, the gross structure of the protein having been destroyed,

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the rates were about the same. The total hydrolysis of the native protein was greater by an amount equivalent to 10 peptide linkages per molecule of 35,000.* The difference became apparent only when additional amounts of fresh enzyme were added to insure complete digestion. This behavior is representative of all the studies made with the egg albumins.

TABLE I
Characteristics of Derived Proteins

Note that in this paper, as well as in others of this series, CSA refers to carbon suboxide-treated serum albumin, CEA to carbon suboxide-treated egg albumin, and CO₂-EA to carbon dioxide-treated egg albumin, the number suffixed denoting the particular preparation. In this way CSA-6 has been employed in earlier publications.

Preparation*	CSA 6	CEA-4	CO ₂ EA soluble fraction
Original amino N per gm. atom N, mole	0.080	0.042	0.042
Reagent added per mole of reactive groups, moles	3.2	1.7	3.3
Total combined malonyl per gm. atom N, mole	0.080	0.039	
Ratio, final amino to total N	0.008	0.008	0.042

* All additions were made at pH 7.5 to 7.6.

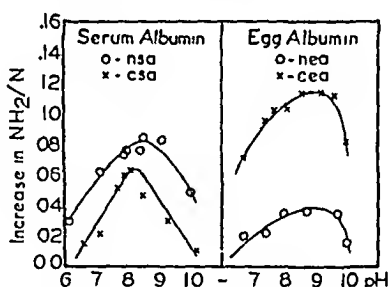


FIG. 1. Effect of pH upon the chymotryptic digestions of native serum albumin (*nsa*), malonyl serum albumin (*csa*), native egg albumin (*nea*), and malonyl egg albumin (*cea*). The time for the serum albumin experiments was 6.5 hours, for the egg albumin experiments 3.5 hours.

Native serum albumin was also more completely degraded than malonyl serum albumin, the difference representing about 40 linkages per molecule of 71,000. The behavior of the two forms, native and malonyl, was not consistent, however, as regards the initial stages of digestion. The data plotted in Fig. 3 show that the native protein is more rapidly digested even at the beginning of the reaction; this was more frequently observed, but

on at least one occasion carbon suboxide-treated serum albumin was more rapidly digested than the untreated form.

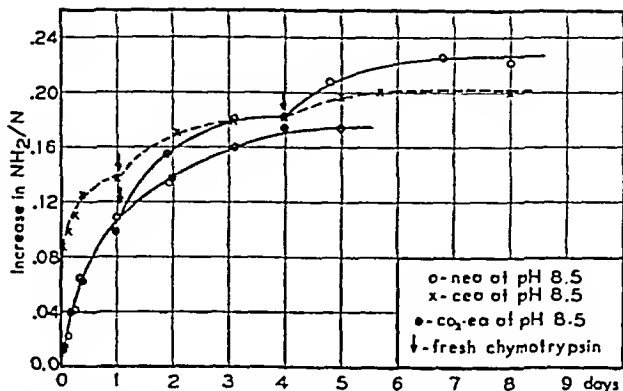


FIG. 2. Exhaustive chymotryptic digestions of native (O), malonyl (X), and CO₂-treated egg albumin (●). The substrate concentration was 3.5 mg. of N per ml. and the initial enzyme concentration was 0.19 mg. of N per ml., subsequent additions being the same.

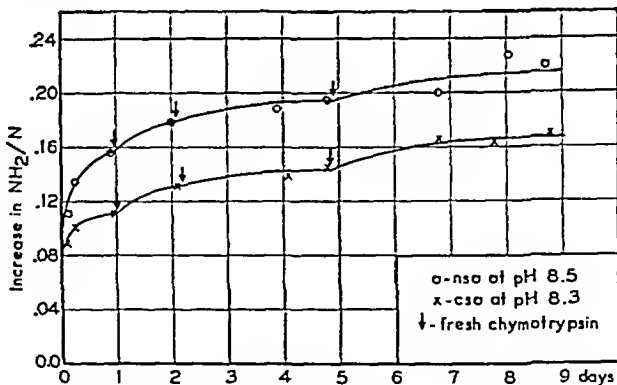


FIG. 3. Exhaustive chymotryptic digestions of native (O) and malonyl serum albumin (X). The substrate concentration was 4.04 mg. of N per ml. and the initial enzyme concentration was 0.58 mg. of N per ml., subsequent additions being 0.30 mg. of N per ml.

Several factors may be involved in determining the initial digestion rates of the various proteins studied. It was thought that physical denaturation might have taken place at the gas-liquid interface during the

addition of gaseous carbon suboxide to the aqueous protein solution. Such denaturation would be analogous to that caused by violent shaking of egg albumin solutions, which has been studied by Bull and Neurath (5) and by Wu and Wang (6). In order to test this possibility, a solution of egg albumin was treated with carbon dioxide in the same apparatus and under exactly the same conditions, temperature, pH, and rate of gas flow, as were used for the addition of carbon suboxide. During the purification of the product there was formed some insoluble material which was discarded, as all of the treated proteins thus far employed have been freely soluble in water near their isoelectric points. Parallel digestions of the soluble CO₂-treated egg albumin and native egg albumin failed to reveal any differences which might be attributed to denaturation (Fig. 2).

Another factor which might influence the rates of digestion is the antitryptic substance of raw egg white, investigated by Balls and Swenson (7, 8). There appears to be no evidence denoting which enzyme of crude pancreatic extract this substance affects. If egg white antitrypsin should inhibit chymotrypsin and if some of it were still associated with our crystalline egg albumin, its removal during the preparation of malonyl egg albumin or inactivation by carbon suboxide might account for the greatly accelerated hydrolysis of malonyl egg albumin. The occurrence of a similar trypsin inhibitor in the serum of the horse appears not to have been investigated.

The concentrated egg white inhibitor (Preparation E) was prepared and its effect upon the chymotryptic hydrolysis of casein was studied. Balls and Swenson found that, under their conditions, 0.04 mg. per ml. reduced the hydrolysis of casein by crude pancreatin to the extent of 50 per cent. We observed that 8.0 mg. per ml., that is 200 times as much, reduced the chymotryptic hydrolysis of casein by only 15 per cent. Crude thin white of egg was also studied; its inhibitory effect upon chymotrypsin was of the same degree.

The extent of the 1 hour hydrolysis of native egg albumin in the experiments of Fig. 2 is only 14 per cent of that of the malonyl protein. If this difference is interpreted as inhibition, the inevitably small amount of inhibitor still associated with the crystalline protein has an effect equal to 86 per cent. This would appear to be wholly unlikely.

We have therefore concluded, in regard to this behavior, that the carbon suboxide treatment frequently results in a partial opening up of the protein molecule, so that it is more available to the enzyme during the initial stages of digestion. The more labile state is characteristic of all our malonyl egg albumins but is relatively rare with the corresponding serum albumins. Serum albumin is generally a more stable protein than egg albumin.

Our experimental data consistently indicate that both native egg and

serum albumins are further hydrolyzed by chymotrypsin than are the corresponding malonyl proteins. Since the final extent of hydrolysis is largely dependent upon the nature of the polypeptide molecules which constitute the system at this stage, this influence must be attributed to the presence of malonyl residues on these polypeptide chains rather than to the effect of malonylation upon the gross structure of the protein molecule itself. Carbon suboxide is known to react with phenolic hydroxyls of tyrosine and with free amino groups. Of the two types of linkage thus formed, the first or O-malonyl is slowly hydrolyzed in nearly neutral aqueous solution even at refrigerator temperatures (9). It is much more unstable at 37° (Fig. 4¹), so that there would be no significant amount of malonic acid bound to tyrosine after the 8 days of digestion under these

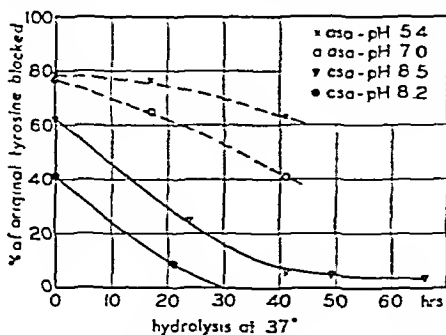


FIG. 4. Hydrolysis of O-malonyl groups in malonyl serum albumin (csa) and O-acetyl in acetyl serum albumin (asa) under conditions of enzymic hydrolysis. The liberation of phenolic hydroxyl was determined by Herriott's method (10).

conditions. The effect of the carbon suboxide treatment, therefore, may not be attributed to malonyl groups on tyrosine but rather to malonyl on lysine, where it is stable, or to malonyl bound in some other way which we are not in a position to consider.

This conclusion is surprising in view of the fact that at the present time, chymotrypsin is known to hydrolyze only polypeptides which contain tyrosine or phenylalanine residues, such as carbobenzoxytyrosylglycinamide (11). Lysine-containing substrates are attacked by trypsin (12). The possibility that our chymotrypsin preparation might have been contaminated with trypsin was eliminated by assays with α -hippuryllysineamide

¹ Hydrolysis of the O-acetyl group is also quite appreciable at 37° (Fig. 4). Acetyl serum albumin is slowly hydrolyzed at low temperatures; a preparation having 79 per cent of the phenolic hydroxyls originally covered lost 8 per cent of its O-acetyl after 27 days and 19 per cent after 77 days at 4° and pH 5.2.

which is very readily hydrolyzed by trypsin. These indicated that there was no trypsin present, or at the most a negligible amount. The results suggest that some groups other than aromatic residues are in part responsible for the digestion of proteins by chymotrypsin.

This idea is supported by correlating the number of linkages split with the tyrosine and phenylalanine contents of the proteins studied. About 172 linkages are hydrolyzed per molecule of native serum albumin. The best available analyses indicate 18 tyrosine (2) and 33 phenylalanine residues (13), giving a total of 51 to be compared with 172 linkages split. A similar incongruity is shown by normal egg albumin, in which 89 linkages are hydrolyzed in contrast to 20 aromatic amino acid residues (8 tyrosine and 12 phenylalanine) (14).

The preliminary experiments were carried out with the collaboration of Dr. H. N. Christensen, to whom the authors wish to express their gratitude.

EXPERIMENTAL

pH Optima Tests—For the determination of the pH optimum for the digestion of the egg albumins, the concentrations of substrate and enzyme were 3.06 and 0.19 mg. of nitrogen per ml., respectively; for the serum albumins the respective concentrations were 4.03 and 0.58 mg. per ml. The solutions were 0.15 M in phosphate or borate buffers, and digestions were carried out at 37.5°. Throughout this work the extent of protein hydrolysis was followed by determining the increase in amino nitrogen with the volumetric Van Slyke apparatus (Koch's modification).

Exhaustive Chymotryptic Digestions—The solutions for these experiments were 0.15 M in borate buffer and contained the concentrations of substrate and enzyme specified below Figs. 2 and 3. Incubation was carried out at 37.5°. Fresh chymotrypsin was added at intervals as indicated by the arrows.

Studies with Egg White Inhibitor—The concentrated inhibitor, Preparation E, was prepared from egg white (7). The crude inhibitor solution, which was also investigated, consisted of the thin white from three eggs diluted with 1 volume of 0.6 M phosphate buffer of pH 7.9. The solution contained 8.7 mg. of nitrogen per ml.

The digests, at pH 8.0, were made up to contain 30 mg. of casein (according to Hammarsten) per ml., 0.25 mg. of chymotrypsin nitrogen per ml., and the inhibitor. The inhibitor and casein solutions were thoroughly mixed at 37.5° before addition of the enzyme, and digestion was continued for 40 minutes at this temperature. Enzyme, substrate, and inhibitor controls were maintained.

These conditions differ from those employed by Balls and Swenson in their study of the antitryptic effect, but were chosen to follow more closely the conditions we employed in studying the exhaustive chymotryptic digestion of egg albumin.

The results are summarized in Table II.

TABLE II
Effect of Egg White Antitrypsin upon Chymotryptic Digestion of Casein

Inhibitor	Increase in amino N per 2 ml.*	Per cent inhibition
	mg.	
None.....	0.81	
8 mg. Preparation E per ml. digest.....	0.69	15
0.25 ml. crude inhibitor solution per ml. digest.....	0.65†	20
0.25 " " " " " " " ".....	0.81‡	0

* Corrected for any action of chymotrypsin on inhibitor.

† Average of four digestions.

‡ Casein and the inhibitor were incubated 40 minutes at 37.5° before addition of the enzyme.

Tests for Presence of Trypsin—The assay of crystalline chymotrypsin followed the procedure of Hofmann and Bergmann (12) for studying the specificity of trypsin. The concentration of α -hippuryl-L-lysineamide hydrochloride was 0.05 mM per ml. in 0.15 M phosphate buffer of pH 7.2. The temperature was 40°. Hydrolysis was followed by titration of liberated carboxyl groups by the method of Grassmann and Heyde (15). The concentration of chymotrypsin nitrogen was 0.2 mg. per ml., such that the presence of 1.2 per cent contaminating trypsin would cause 50 per cent hydrolysis in 24 hours. Enzyme and substrate controls were maintained.

No hydrolysis was observed in 21 hours, and in 52 hours only 3 per cent occurred.

BIBLIOGRAPHY

1. Ross, W. F., and Christensen, H. N., *J. Biol. Chem.*, **137**, 89 (1941).
2. McMeekin, T. L., *J. Am. Chem. Soc.*, **61**, 2884 (1939).
3. Kekwick, R. A., and Cannan, R. K., *Biochem. J.*, **30**, 227 (1936).
4. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1935).
5. Bull, H. B., and Neurath, H., *J. Biol. Chem.*, **118**, 163 (1937).
6. Wu, H., and Wang, C.-F., *J. Biol. Chem.*, **123**, 439 (1938).
7. Balls, A. K., and Swenson, T. L., *J. Biol. Chem.*, **106**, 409 (1934).
8. Balls, A. K., and Swenson, T. L., *Ind. and Eng. Chem.*, **26**, 570 (1934).
9. Tracy, A. H., and Ross, W. F., *J. Biol. Chem.*, **142**, 871 (1942).

10. Herriott, R. M., *J. Gen. Physiol.*, **19**, 283 (1935-36).
11. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 405 (1937).
12. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **130**, 81 (1939).
13. Block, R. J., and Bolling, D., *The determination of the amino acids*, Minneapolis, revised edition (1940).
14. Bernhart, F. W., *J. Biol. Chem.*, **132**, 189 (1940).
15. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).

DETERMINATION OF NICOTINIC ACID IN BLOOD CELLS AND PLASMA*

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A number of chemical methods have been proposed for the assay of nicotinic acid in blood (1-8). The concentration of nicotinic acid in blood cells is at least 10 times that in the plasma (9, 10). In general, the interpretation of changes in the concentration of a substance in whole blood is difficult and dubious when the concentration of the substance in cells and plasma is markedly different; *e.g.*, chloride. Despite the possible ambiguity in interpretation of results, the methods described have been directed primarily to the analysis of whole blood.

In the assay of nicotinic acid in blood, cells and plasma are considered separately. The method described for the cells seems satisfactory with respect to the usual criteria; *i.e.*, recovery of added material, reproducibility of results, simplicity, and economy of sample. The method described for plasma also seems satisfactory with respect to the first two requirements. It involves, however, more manipulations, and the quantity of plasma needed, at least 10 ml., will limit its use. Both procedures contain adaptations of methods that have been employed in the analysis of blood (1, 5) and other material (11, 12). Some data on human and dog blood are given.

EXPERIMENTAL

Assay of Nicotinic Acid in Blood Cells—2 ml. of cells obtained from oxalated blood are placed in a 20 × 150 mm. Pyrex test-tube, and 12 ml. of water added. The cells and water are mixed and allowed to stand 5 to 10 minutes. 3 ml. of 0.67 N sulfuric acid are then added, and, after mixing, 3 ml. of 0.303 M sodium tungstate (10 gm. of $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ per 100 ml. of solution). The mixture is vigorously shaken and then placed in a boiling water bath for 20 minutes. The contents of the tube are mixed frequently during the first 5 minutes, and occasionally thereafter, to prevent the precipitate from collecting at the top of the fluid and being forced out of the tube. When the material is not being mixed, the top of the tube should be covered in order to limit evaporation. The device illustrated in Fig. 1

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serves as a convenient mixer and cover. The mixture is cooled to room temperature, centrifuged, and the supernatant liquid filtered through a 5 cm., No. 501, Sargent filter paper.

10 ml. of filtrate are transferred to an 18×150 mm. Pyrex tube calibrated at 20 ml. 2 ml. of concentrated hydrochloric acid are added. The solution is heated $1\frac{1}{2}$ hours in a boiling water bath. After cooling, a drop of alcoholic phenolphthalein, 0.5 gm. per 100 ml., and sufficient potassium hydroxide solution, about 11 M, are added to make the solution just alkaline

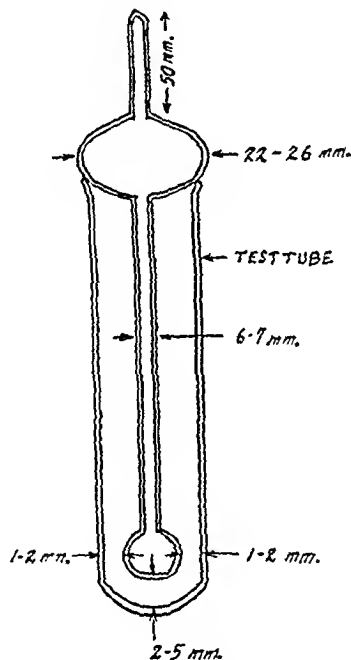


FIG. 1. Mixer and cover. Mixing is effected by raising and lowering the device which is made from Pyrex glass tubing.

to phenolphthalein. It is convenient to adjust the alkali so that the addition of some convenient volume, *e.g.* 2 ml., will almost complete the neutralization, the further adjustment being made by dropwise additions of alkali. The solution is then adjusted to about pH 5 by the dropwise addition of 4 M hydrochloric acid, Alkacid paper¹ being used to estimate the pH. 2 ml. of 1.6 M potassium dihydrogen phosphate are added. The solution is then diluted to 20 ml.

The nicotinic acid in the solution is estimated by means of the color produced by its reaction with cyanogen bromide and *p*-methylaminophenol

¹ Supplied by the Fisher Scientific Company.

sulfate (elon). The measurements of color are made with the Evelyn photoelectric colorimeter with Filter 400. The general details of the application of this reaction have been adequately described (11, 12). The pertinent details for the present analysis are the following. 9 ml. aliquots of the adjusted hydrolysate are placed in each of two colorimeter tubes. To one, which serves as a blank, are added 10 ml. of water and 1 ml. of 0.35 N sulfuric acid. To the other are added 1 ml. of cyanogen bromide, and, after heating as described (11), 10 ml. of aminophenol solution.

The optical density of the color of the blank is subtracted from the optical density of the color of the treated sample. The nicotinic acid in the sample is estimated by comparing the difference obtained with the optical density of the color obtained from known amounts of nicotinic acid treated in the same fashion as the hydrolysate.

The color of the blank is about 20 per cent of that due to nicotinic acid. The ideal blank would, of course, contain no color. A blank color introduces an unavoidable instrumental error, and the possibility of error due to reactions of the colored material with the reagents. Such reactions might affect the final color apparently due to nicotinic acid in all possible ways. The following tests indicate that the material producing the blank color probably does not introduce errors other than instrumental, and that the corrected measure of color is due to nicotinic acid. The ratio of the corrected optical density obtained with Filter 400 to that obtained with Filter 420 was the same for the sample and nicotinic acid. The ratio of the corrected optical density of the color produced by the reaction of the test material and cyanogen bromide alone to that of the corrected optical density of the color produced in the complete reaction was the same for the sample and nicotinic acid. Samples of the hydrolysates were treated with Lloyd's reagent and lead nitrate in a fashion similar to the treatment described below for plasma and described previously for other material (11, 12). This treatment decreases the blank by about one-half. The results obtained by the more elaborate treatment were practically the same as those obtained with the procedure given.

The reproducibility of results is reasonably good. The maximum difference between duplicates was 15 per cent. The average difference was about 5 per cent. The recovery of nicotinic acid, added in the form of solutions of the free acid, nicotinamide, and pyridine nucleotides, was between 90 and 100 per cent. The amount of added material was sufficient to increase the original concentration of nicotinic acid about 20, 50, and 100 per cent.

Assay of Nicotinic Acid in Blood Plasma—A protein-free filtrate is prepared from a mixture of 10 ml. of plasma plus 2 ml. of water by the treatment described under the analysis of cells. 10 ml. of filtrate are hydrolyzed

with 2 ml. of concentrated hydrochloric acid for $1\frac{1}{2}$ hours in a 16×150 mm. Pyrex tube calibrated at 12 ml. The cooled hydrolysate is diluted to the mark, mixed, and centrifuged. To 11 ml. of the supernatant fluid, in a 16×150 mm. tube calibrated to hold 7 ml. of solution and 0.5 gm. of Lloyd's reagent, is added 1 ml. of approximately 11 M potassium hydroxide solution. The alkali should be adjusted so that 1 ml. will bring the pH of the hydrolysate between 0.5 and 1.0. 0.5 gm. of Lloyd's reagent is added. The contents of the tube are carefully mixed in order to liberate gas, and then mixed thoroughly by shaking. The Lloyd's reagent is washed down with a few drops of 0.2 N sulfuric acid. The tube is centrifuged and the supernatant liquid discarded. The Lloyd's reagent is washed in the centrifuge with 2 ml. of 0.2 N sulfuric acid. 4 ml. of about 0.5 M potassium hydroxide are added to the Lloyd's reagent. The materials are mixed thoroughly. 1 drop of phenolphthalein is added and followed by water to the mark. The supernatant fluid, collected by centrifugation, is added to and thoroughly mixed with 0.3 gm. of powdered lead nitrate. The alkali preferably should be adjusted so that the mixture is not quite alkaline to phenolphthalein after the treatment with lead nitrate. The mixture is centrifuged and the supernatant liquid collected. Solid tripotassium phosphate is added to the supernatant liquid with mixing until the liquid is just alkaline to phenolphthalein. The supernatant liquid is collected by centrifugation and 5 ml. of the liquid are transferred to a tube calibrated at 9.5 ml. The solution is then adjusted to about pH 5 by the dropwise addition of 4 M hydrochloric acid, Alkacid paper being used to estimate the pH. 0.9 ml. of 1.6 M potassium dihydrogen phosphate is then added, followed by water to the mark. 4.5 ml. aliquots are transferred to each of two colorimeter tubes and treated with 0.5 volume of the reagents as directed under the assay in blood cells. The corrected optical density of the unknown is compared with that produced by 0.5, 1.0, and 2.0 γ of nicotinic acid carried through all stages of the analysis except the treatment with tungstic acid.

The optical densities of the color produced by the nicotinic acid carried through as standards when compared with the densities of the color produced by equivalent quantities of nicotinic acid treated directly with the reagents indicate a loss of less than 10 per cent due to the manipulations involved.

The blank color is about one-third that obtained from the unknown. The maximum difference between duplicates was 25 per cent, the average difference 15 per cent. Recovery of added nicotinic acid, nicotinamide, and pyridine nucleotide added to the plasma was from 80 to 120 per cent. Considering the number of manipulations and the relatively small quantity

of nicotinic acid handled, the agreement between duplicates and the recovery of added material are as good as may be expected.

Comments on Procedures—The use of tungstic acid filtrates in the assay of nicotinic acid in blood has been proposed (1, 3, 5). With filtrates prepared as previously described (3, 5), recovery of added nicotinic acid, amide, and pyridine nucleotides from blood cells was adequate. However, heating the precipitated material as indicated above increased the yield of nicotinic acid by 20 per cent. In addition, hydrolysis with 2 N hydrochloric acid for 1½ hours produced the maximum final color and yielded 10 per cent

TABLE I

Concentration of Nicotinic Acid in Blood Cells and Plasma of Dogs after Intravenous Dosage with Nicotinic Acid

State of animal	Dose	Concentration of nicotinic acid						
		Cells				Plasma*		
		Time after dose				Time after dose		
		0 min.	30 min.	60 min.	120 min.	30 min.	60 min.	120 min.
	mg. per kg.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.
Normal	3	15	15	20	20	0	0	0
"	10	16	24	21	17	19	10	3
"†	10	20	24	24	21	11	9	2
"†	10	16	24	18	19	7	2	1
"	12	19	24	22	19	18	4	2
"	16	18	31	26	22	24	10	2
Blacktongue	10	19	25	19	23	10	6	1
"	10	21		19	21		3	2
"	18	13	29	29	24	30	25	8

* The values given represent the differences between the results obtained after dosage and the result obtained before dosage. The plasma filtrates were analyzed by the procedure used for the analysis of cells, as indicated in the text.

† Received 250 mg. of nicotinic acid per day for 1 week preceding the test.

more color than was obtained under the conditions previously described (3, 5).

An attempt was made to analyze plasma filtrates in the same fashion as cell filtrates. Recovery of added material was reasonably satisfactory. However, the colors produced by the hydrolyzed plasma filtrates and cyanogen bromide alone indicated quantities of nicotinic acid 3 or 4 times greater than the amounts indicated by the complete reaction. It is possible that the extra color produced with cyanogen bromide does not affect the final color, but it must be assumed that it does. That it probably does affect the final color is suggested by the fact that the results obtained with the

hydrolyzed filtrates directly were several times the results obtained by the procedure described above.

Although the analysis of plasma filtrates by the procedure used for the cells was not satisfactory for the measurement of normal levels, the procedure does serve to describe the level as affected by the ingestion of relatively large quantities of nicotinic acid. The initial value obtained on the plasma serves as a base-line. For this purpose 3 to 5 ml. of plasma, depending upon the amount of nicotinic acid given, were treated with tungstic acid, the filtrates hydrolyzed and centrifuged as described above, and the hydrolysates treated as described for the assay of cells. Data obtained in this way are given in Table I.

Application of the Lloyd's reagent and lead nitrate treatment (11) to acid and alkaline hydrolysates of blood cells and plasma was not successful. Recovery of added nicotinic acid and its derivatives was low. Since this treatment has been applied successfully to other materials, the low recoveries suggest that nicotinic acid may be destroyed when heated with strong acid in the presence of blood. The preparations obtained by the treatment of acid hydrolysates with Lloyd's reagent and lead nitrate, charcoal (7), and zinc hydroxide (6) gave results for cells of the same order of magnitude as those obtained by the method described above. However, all of these treatments produced final solutions that reacted with cyanogen bromide alone to give colors indicating considerably more nicotinic acid than was indicated by the final complete color reaction obtained with cyanogen bromide and the aminophenol. It seems possible that loss of nicotinic acid during hydrolysis of cells may be compensated for by the production of color from a reaction of non-nicotinic acid material and cyanogen bromide.

Results

Data obtained on the cells of human and dog blood are given in Table II. The pellagrin was described clinically as having typical symptoms that responded to nicotinic acid therapy. The deficiency states were described as general with no predominating feature. Blacktongue in the dogs was characterized by the usual symptoms, anorexia, loss of weight, reddening or necrosis of the mucous membranes of the mouth, and salivation. The data indicate that the concentration of nicotinic acid in the blood cells is not affected appreciably by the dietary state. This is in agreement with the observations that the pyridine nucleotide concentration of the cells is not appreciably affected in pellagra or blacktongue (13-15). Agreement between the conclusions drawn from analysis of nicotinic acid and pyridine nucleotides is to be expected, since, as shown by the data in Table III, the pyridine nucleotides account for the major portion of the nicotinic acid in human cells.

The effect of an intravenous dose of nicotinic acid on the level of nicotinic acid in the blood cells and plasma of normal dogs and dogs with blacktongue is shown in Table I. No differences, interpretable in terms of the deficiency, were found. Similarly, it has been found that the blood and plasma levels of nicotinic acid in pellagra after dosage with nicotinic acid are not appreciably different from those of normals (9).

TABLE II

Concentration of Nicotinic Acid in Blood Cells of Human Subjects and Dogs

Subject	State of subject	No. of subjects	Concentration of nicotinic acid	
			Mean	Range
			γ per ml.	γ per ml.
Human	Normal	8	13	12-15
"	Pellagrous	1	12	
"	Deficiency	7	14	12-16
Dog	Normal	10	16	13-20
"	"	2	18	16-20
"	Blacktongue	10	19	13-23

* 250 mg. of nicotinic acid were given daily for 7 days preceding removal of the samples for analysis.

TABLE III

Concentration of Nicotinic Acid and of Pyridine Nucleotides in Human Blood Cells

Sample No.	Concentration of nicotinic acid	Concentration of pyridine nucleotides* in terms of nicotinic acid
	γ per ml.	γ per ml.
1	10	9
2	9	8
3	10	9
4	12	10

* Measured as factor V (13, 16).

The average concentration of nicotinic acid in the plasma of six normal human subjects was 0.3 γ per ml. The range was 0.2 to 0.5 γ per ml. These values are of the same order of magnitude as those obtained by another chemical method (9) and by a microbiological method (10).

SUMMARY

Methods for the determination of nicotinic acid in blood cells and plasma are described. The average concentration of nicotinic acid in the cells of normal man and dogs was 13 and 16 γ per ml. respectively. The pyridine nucleotides account for most of the nicotinic acid in the human cells. The

average concentration of nicotinic acid in the plasma of normal, human subjects was 0.3 γ per ml. The concentration of nicotinic acid in the blood cells was not decreased in blacktongue or pellagra.

BIBLIOGRAPHY

1. Swaminathan, M., *Indian J. Med. Research*, **26**, 427 (1938).
2. Ritsert, K., *Klin. Woch.*, **18**, 934 (1939).
3. Pearson, P. B., *J. Biol. Chem.*, **129**, 491 (1939).
4. Kodicek, E., *Biochem. J.*, **34**, 712 (1940).
5. Stotz, E., *J. Lab. and Clin. Med.*, **26**, 1042 (1941).
6. Friedemann, T. E., and Barborka, C. J., *J. Biol. Chem.*, **138**, 785 (1941).
7. Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, **134**, 1 (1940).
8. Patton, E. W., Sutton, W. R., and Youmans, J. B., *J. Clin. Inv.*, **19**, 785 (1940).
9. Field, H., Jr., Melnick, D., Robinson, W. D., and Wilkinson, C. F., Jr., *J. Clin. Inv.*, **20**, 379 (1941).
10. Isbell, H., Wooley, J. G., Butler, R. E., and Sebrell, W. H., *J. Biol. Chem.*, **139**, 499 (1941).
11. Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, **136**, 729 (1940).
12. Dann, W. J., and Handler, P., *J. Biol. Chem.*, **140**, 201 (1941).
13. Kohn, H. I., *Biochem. J.*, **32**, 2075 (1938).
14. Axelrod, A. E., Spies, T. D., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 667 (1941).
15. Kohn, H. I., Klein, J. R., and Dann, W. J., *Biochem. J.*, **33**, 1432 (1939).
16. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **140**, 739 (1941).

THE EFFECTS OF ADMINISTRATION OF GLUCOSE AND INSULIN ON BLOOD PYRUVATE AND LACTATE IN DIABETES MELLITUS

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The importance of pyruvic and lactic acids in intermediate carbohydrate metabolism has made desirable a study of the behavior of these substances in diabetes mellitus. A number of years ago (1) evidence was presented which suggested that administration of either glucose or insulin produced increased concentrations of lactate in diabetic blood. Other investigations (2-6) indicated that any effect of the injection of insulin only on blood lactic acid in diabetes mellitus might be due to muscular activity accompanying hypoglycemia. While the present experiments were under way, Bueding *et al.* (7) reported that in diabetic subjects and depancreatized dogs no increase in blood pyruvate occurred following the administration of glucose alone, whereas increases took place after the administration of glucose and insulin.

This study deals with pyruvic and lactic acid levels in the blood of diabetic patients while resting and in the postabsorptive state, and with the changes that occur following glucose and insulin given separately and together.

EXPERIMENTAL

Pyruvic acid was determined by the method recently reported from this laboratory (8). Lactic acid analyses were carried out by either the Friedemann-Graesser (9) or Barker-Summerson (10) procedure. A modification of the Folin method (11) was used for the determination of blood sugar.

Blood drawn from one of the arm veins was immediately transferred to a test-tube containing sodium oxalate and sufficient sodium iodoacetate to make a concentration of 0.3 to 0.4 per cent of the latter. Most specimens were collected without stasis, but when there was stasis it was minimal. Preparation of the protein-free filtrate was begun 1 to 2 minutes after the blood was taken. The sulfuric acid and tungstate solutions were added slowly, and the mixture was allowed to stand 10 minutes before centrifugation and filtration.

The diabetic subjects employed in these experiments were patients from

the Division of Metabolic Diseases, who with two exceptions to be noted received their last dose of insulin 14 to 24 hours previous to the test. Most of them were in the process of being standardized with both plain and protamine-zinc insulin. In the study of the effects of glucose and insulin, the first blood specimen was collected when the subject had been at rest for at least 45 minutes and was fasting. He was kept quiet and in bed throughout the test. Glucose was given by mouth as a 15 per cent solution in amounts of 1 gm. per kilo of body weight. Specimens were then taken 0.5, 1, 2, and 3 hours later. In the experiments with insulin alone the 3 hour blood was omitted in an attempt to avoid any increases which could be attributed to increased muscular activity accompanying hypoglycemia (2-5).

Two series of control experiments were also done. Pyruvate changes were determined in normal subjects after glucose ingestion, and variations in both pyruvate and lactate were observed in diabetic subjects when neither glucose nor insulin was given. All subjects were non-febrile.

Statistical Analysis—The effects of glucose and insulin on blood pyruvate and lactate were evaluated statistically. *P* values less than 0.05 were considered significant. These were computed by the methods for small samples according to Mainland (12) by Fisher's table of *t* values (13). Two different comparisons were made: (a) the significance of the mean changes in concentration from the basal values, (b) the significance of the difference between mean changes in concentration of the same substance in two different groups.

Results

The mean concentration of blood pyruvic acid, under basal conditions, for twenty normal subjects was 0.76 ± 0.173 (standard deviation), and of lactic acid for thirteen of this group was 8.9 ± 2.04 mg. per cent. The normal range for pyruvic acid in blood by the Bueding-Wortis method (14) was reported (15) as 0.77 to 1.23 (mean, 1.02) mg. per cent. Lactic acid values were slightly higher than those obtained when glycolysis was completely inhibited (16). With the diabetic group normal basal levels (the mean of the normal group ± 2 times the standard deviation) were observed for pyruvic acid in twenty-seven of twenty-eight subjects, and for lactic acid in twenty-three of twenty-five subjects studied. Bueding, Wortis, and Stern (15) also reported normal basal pyruvate values in diabetes mellitus.

Subject 58 with elevated lactic acid values of 17.3 (Fig. 3)¹ and 14.8 (Fig. 5) mg. per cent showed impaired liver function by the bromosulfalein

¹ Refers to the corresponding pyruvic acid value of this subject.

test. The pyruvate and lactate levels of Subject 65 were found to be 2.65 (Fig. 3) and 35.5, and 2 weeks later, 1.98 and 20.3 mg. per cent. Evidence of impaired liver function in this patient was provided by the subnormal ratio of esterified to total cholesterol in blood serum (47 and 48 per

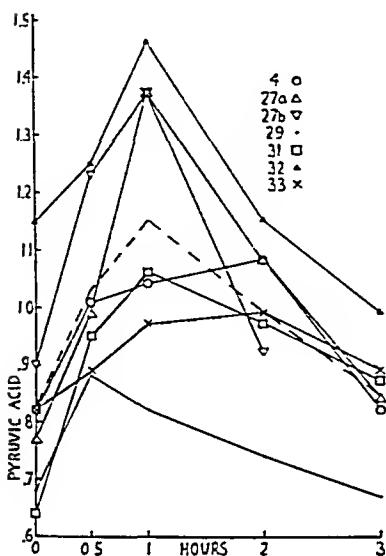


FIG. 1

FIG. 1. Blood pyruvic acid (mg. per cent) in normal subjects following glucose ingestion (mean values, broken line). The values for Subject 27 (a and b) were obtained 3 months apart.

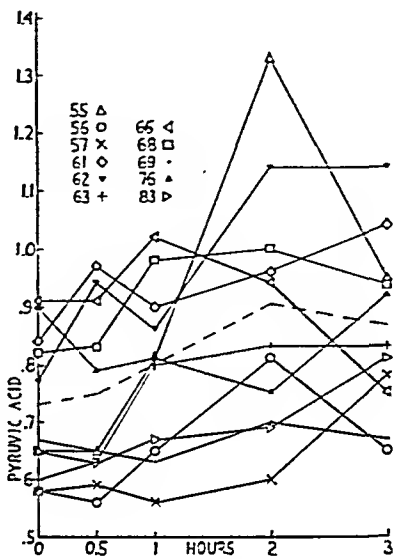


FIG. 2

FIG. 2. Blood pyruvic acid (mg. per cent) in diabetic subjects following glucose ingestion (mean values, broken line). The corresponding blood sugar values (mg. per cent) were as follows: mean values, 156, 220, 275, 300, 272; Subject 55, 130, 218, 290, 290, 280; Subject 56, 90, 150, 254, 300, 232; Subject 57, 116, 178, 228, 240, 220; Subject 61, 165, 169, 187, 259, 270; Subject 62, 243, 346, 377, 415, 400; Subject 63, 117, 180, 259, 235, 166; Subject 66, 146, 214, 292, 296, 240; Subject 68, 136, 205, 228, 179, 156; Subject 69, 168, 227, 268, 330, 315; Subject 76, 300, 352, 414, 454, 406; Subject 83, 93, 192, 243, 295, 304.

cent). This subject was a young female diabetic who exhibited alternate episodes of insulin shock and acidosis.

The effects of administration of glucose and insulin are shown in Figs. 1 to 5.

Blood pyruvate curves in normal subjects (Fig. 1) are in agreement with those observed by Bueding, Stein, and Wortis (17). Increases above the

basal levels were found to be significant at the 0.5 hour ($P < 0.01$), 1 hour ($P < 0.01$), and 2 hour ($P < 0.02$) periods.

In the tests carried out on the diabetic subjects variations in blood pyruvate were accompanied by simultaneous and similar changes in blood lactate. Thus it was found that with the four groups studied the coefficients of correlation between changes in pyruvate and lactate following

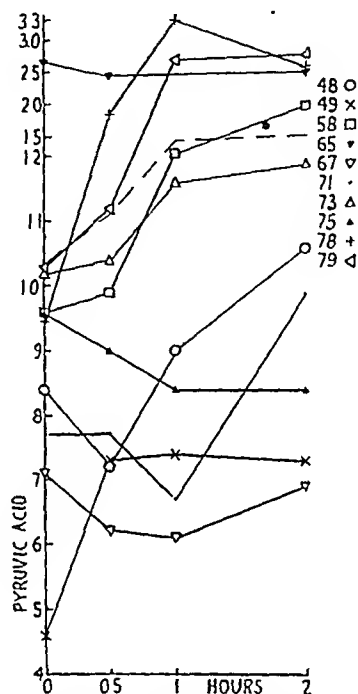


FIG. 3. Blood pyruvic acid (mg. per cent) in diabetic subjects following insulin injection (mean values, broken line). The corresponding blood sugar values (mg. per cent) were as follows: mean values, 252, 244, 226, 179; Subject 48, 142, 132, 119, 82; Subject 49, 146, 143, 131, 96; Subject 58, 300, 300, 270, 220; Subject 65, 466, 444, 420, 360; Subject 67, 130, 136, 130, 110; Subject 71, 248, 238, 228, 195; Subject 73, 232, 218, 218, 178; Subject 75, 142, 150, 148, 126; Subject 78, 348, 304, 262, 170; Subject 79, 364, 376, 331, 256.

treatment and the lactate to pyruvate ratios were as follows: no treatment (Table I), 0.903 and 8.9 ± 0.9 ; glucose (Fig. 2), 0.773 and 11.2 ± 2.3 ; insulin (Fig. 3), 0.932 and 11.5 ± 2.8 ; glucose and insulin (Figs. 4 and 5), 0.912 and 12.4 ± 3.7 . This close relationship has made it unnecessary to report all the blood lactate changes in detail. The mean lactate to pyruvate ratio of 11.3 obtained for all the diabetics under observation is very

close to the value reported by Stotz and Bessey (18) for normal subjects with blood lactic acid levels below 20 mg. per cent.

After the ingestion of glucose by diabetic subjects (Fig. 2), significant pyruvate increases occurred at the 1 hour ($P < 0.05$), 2 hour ($P < 0.05$), and 3 hour ($P < 0.02$) periods. Yet this rise was delayed when compared

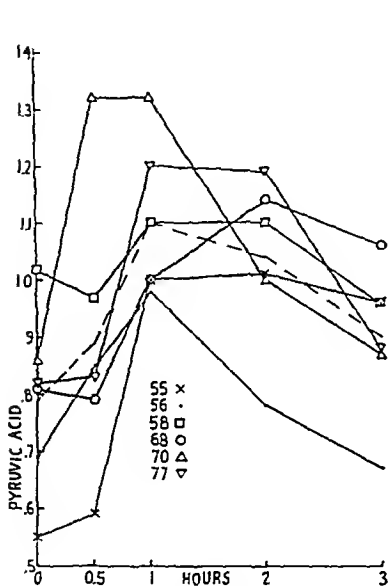


FIG. 4

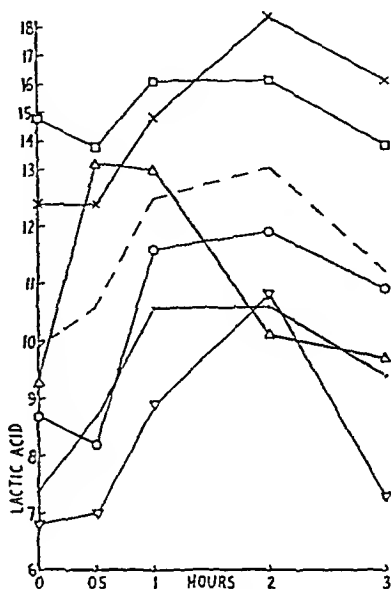


FIG. 5

FIG. 4. Blood pyruvic acid (mg. per cent) in diabetic subjects following simultaneous administration of glucose and insulin (mean values, broken line). The corresponding blood sugar values (mg. per cent) were as follows: mean values, 134, 200, 226, 220, 160; Subject 55, 116, 184, 203, 246, 202; Subject 56, 95, 172, 212, 156, 91; Subject 58, 84, 135, 180, 212, 158; Subject 68, 138, 200, 169, 136, 85; Subject 70, 100, 177, 228, 237, 134; Subject 77, 272, 332, 360, 334, 288.

FIG. 5. Blood lactic acid (mg. per cent) in diabetic subjects following simultaneous administration of glucose and insulin (mean values, broken line). The symbols are identified in Fig. 4.

with that obtained for normal subjects (Fig. 1). Thus at the 0.5 hour ($P < 0.01$) and 1 hour ($P < 0.05$) periods the increases for the normal group were significantly greater than those of the diabetics. In seven of the experiments with diabetic subjects that included determination of both pyruvate and lactate, the increases in either constituent were not significant. With some, the changes did not differ from those observed in

control experiments in which no glucose was given (Table I). These results disagree with those of Bueding *et al.* (7) who found no significant pyruvic acid increase, and with those of Katayama and Killian (1) who reported lactic acid increases, in the human diabetic after ingestion of glucose. The experimental conditions employed by the former² (7) differed in two important details from ours, in that prior to their tests insulin was withheld for 24 to 48 hours and protamine-zinc insulin was discontinued for at least a week. Also included in our group was one subject, No. 63 (Fig. 2), whose diabetes was mild enough to be controlled without insulin. The data presented by Katayama and Killian (1) did not show whether the lactic acid increases could be considered statistically significant.

TABLE I

Variations in Fasting, Resting Levels of Pyruvic Acid, Lactic Acid, and Sugar, in Blood of Diabetic Subjects

The values are given in mg. per 100 cc. of blood.

Subject No.	Constituent of blood analyzed	Time interval after first blood				
		0	0.5 hr.	1 hr.	2 hrs.	3 hrs.
66	Pyruvic acid	0.79	1.02	1.02	1.03	0.98
72	" "	0.95		0.97	1.00	1.03
82	" "	1.04	1.03	0.98	0.92	0.92
84	" "	0.92	1.00	1.02	1.08	1.04
66	Lactic "	6.4	8.9	8.5	8.4	7.9
72	" "	9.1		10.4	8.8	11.0
82	" "	8.9	8.6	8.6	8.3	8.5
66	Sugar	131	139	148	151	166
72	"	271		256	250	245
82	"	228	232	235	243	252
84	"	301	321	304	299	309

The response of pyruvate (Fig. 3) and lactate following the injection of insulin varied widely. A trend toward higher values was apparent for the group. Yet the increases were found to be statistically not significant. The responses in a number of experiments were similar to those obtained when insulin was not given (Table I). The type of lactate and pyruvate curves showed no relationship to the test dose of insulin (5 to 20 units; mean, 10) or to the rate of fall of blood sugar. The greatest rise observed in any subject studied was obtained in three of this group. Their fasting blood sugar levels were 300 mg. per cent or over, and they had received large doses of insulin on the previous day (Subject 58, 175 units; Subject 78, 72 units; Subject 79, 59 units). The latter had been given 10 units of

² Bueding, E., personal communication.

plain insulin 8 hours prior to the test. The absence of significant increases for both pyruvate and lactate, when insulin alone was given and insulin shock was not encountered, agrees with the work on lactate levels reported by other investigators (2-6).

When insulin and glucose were administered together, increases in blood pyruvate (Fig. 4) and lactate (Fig. 5) occurred in all the diabetic subjects tested. The rise was highly significant for pyruvic acid at the 1 hour ($P < 0.01$) and significant at the 2 hour ($P < 0.02$) period, and highly significant for lactic acid at the same two periods ($P < 0.01$ for both). The test dose of insulin used in this series varied from 10 to 50 (mean, 20) units. When the effect of glucose plus insulin was compared with that of glucose alone (Fig. 2) in the diabetic subjects studied, it was observed that at 1 hour the increase in pyruvic acid ($P < 0.01$) and in lactic acid ($P = 0.01$) was significantly greater after glucose plus insulin. A similar comparison of differences in the pyruvate rise made between the latter group (Fig. 4) and the normal subjects (Fig. 1) showed no statistical differences at any of the four periods. The effect of insulin and glucose in producing a significant increase in lactate and a more rapid pyruvate rise in the blood of our diabetic subjects as compared with the effect of glucose alone agrees with the results of the pyruvic acid studies of Bueding *et al.* (7). They reported that in both the human diabetic and the depancreatized dog insulin increased the formation of pyruvate after the administration of glucose. A similar comparison with the observations on lactic acid of Katayama and Killian (1) cannot be made, because their experimental procedure differed from ours in too many details.

DISCUSSION

Our data indicate that insulin in the presence of ingested glucose augmented the formation of both pyruvic and lactic acids in diabetes mellitus. This effect occurred at the same time that insulin increased the utilization of the ingested glucose. The values for the lactate to pyruvate relationship encountered during this time were in that range which Stotz and Bessey (18) suggested might be determined in large part by tissues other than the muscles. It is thus quite possible that the increase in glucose utilization was responsible for the rise in pyruvic and lactic acid levels. These considerations therefore support the suggestion of Bueding, Stein, and Wortis (17) that pyruvic acid is a normal intermediary in the metabolism of glucose in man. The more recent studies of Bueding *et al.* (7) present further evidence for these conclusions.

The non-significant variations which followed the injection of insulin alone do not necessarily exclude the possibility that insulin *per se* is effective in increasing pyruvate and lactate formation. It may be pointed

out that an average of one-half the dose was administered compared with the amount injected when glucose was also given. The use of a greater amount was avoided in order to obviate any significant rise resulting from increased muscular activity.

The difference in pyruvate response after glucose alone in our experiments and in those of Bueding *et al.* (7) may be due to a difference in severity of diabetes, an explanation also suggested by Bueding.² With our subjects the use of protamine-zinc insulin, a slow acting insulin, and the shorter period of withholding insulin prior to the tests may have made more insulin available.

SUMMARY

1. In the human diabetic, fasting and at rest, blood pyruvate levels were normal in twenty-seven of twenty-eight subjects, and blood lactate was normal in twenty-three of twenty-five subjects studied.

2. That a temporary rise in blood pyruvate follows the ingestion of glucose by normal subjects is confirmed.

3. In a group of diabetic subjects, fasting and at rest, and deprived of insulin for from 14 to 24 hours, the following changes in blood lactate and pyruvate were observed. Following glucose ingestion a delayed rise in blood pyruvate and a non-significant increase in blood lactate occurred. Administration of insulin in amounts insufficient to produce physical symptoms of shock was followed by non-significant variations. When both insulin and glucose were administered, significant increases in both metabolites took place.

4. Close correlation between the rate of change in pyruvate and lactate concentrations was noted.

The author wishes to express his appreciation to Dr. John G. Reinhold for advice and criticism, and to the staff of the Metabolic Division for their cooperation.

BIBLIOGRAPHY

1. Katayama, I., and Killian, J. A., *J. Biol. Chem.*, **71**, 707 (1926-27).
2. Briggs, A. P., Kocchig, I., Doisy, E. A., and Weber, C. J., *J. Biol. Chem.*, **58**, 721 (1923-24).
3. Tolstoi, E., Loebel, R. O., Levine, S. Z., and Richardson, H. B., *Proc. Soc. Exp. Biol. and Med.*, **21**, 449 (1923-24).
4. Best, C. H., and Ridout, J. H., *J. Biol. Chem.*, **63**, 197 (1925).
5. Cori, C. F., *J. Biol. Chem.*, **63**, 253 (1925).
6. Valentin, F., *Munch. med. Woch.*, **72**, 86 (1925).
7. Bueding, E., Fazekas, J. F., Herrlich, H., and Himwich, H. E., *Science*, **95**, 282 (1942).

8. Klein, D., *J. Biol. Chem.*, **137**, 311 (1941).
9. Friedemann, T. E., and Graesser, J. B., *J. Biol. Chem.*, **100**, 291 (1933).
10. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941).
11. Folin, O., *J. Biol. Chem.*, **67**, 357 (1926).
12. Mainland, D., *The treatment of clinical and laboratory data*, Edinburgh and London, 149, 156 (1938).
13. Fisher, R. A., *Statistical methods for research workers*, Edinburgh and London, 6th edition, Table IV (1936).
14. Bueding, E., and Wortis, H., *J. Biol. Chem.*, **133**, 585 (1940).
15. Bueding, E., Wortis, H., and Stern, M., *J. Clin. Inv.*, **21**, 85 (1942).
16. Bueding, E., and Goldfarb, W., *J. Biol. Chem.*, **141**, 539 (1941).
17. Bueding, E., Stein, M. H., and Wortis, H., *J. Biol. Chem.*, **137**, 793 (1941); **140**, 697 (1941).
18. Stotz, E., and Bessey, O. A., *J. Biol. Chem.*, **143**, 625 (1942).

THE PREPARATION OF ASPARAGINE

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Asparagine, the β -amide of aspartic acid, is widely used as a source of organic nitrogen in the media upon which certain bacteria and other lower organisms are grown. The greatest single demand for this substance is doubtless for the production of tuberculin, for which Long's synthetic medium (1) is commonly employed. Hitherto, much of the asparagine required has been imported from abroad, but, during the past 2 years, the substance has been difficult to obtain, although moderate supplies have recently appeared on the market.

A careful review of the literature has shown that the information available to one who wishes to prepare a supply of asparagine is curiously meager, since most studies, especially those of Schulze in the period from 1880 to 1900, deal primarily with its physiological relationships. An article by Schulze and Winterstein (2) does indeed give a general account of methods suitable for the preparation of relatively small samples, but assumes a considerable knowledge of, and experience with, the isolation of substances from plants, and omits directions for the production of the etiolated seedlings invariably employed as a source. Murneek (3), a few years ago, reviewed the physiology of asparagine, and brief accounts of the early literature of the substance have been given in two previous papers from this laboratory (4).

The present paper is an attempt to provide the specific information necessary for the laboratory preparation of asparagine in reasonably large quantities. Little is included that was not familiar to Schulze, or even to Piria (5) nearly a century ago, save details of the technique.

EXPERIMENTAL

Selection of Seeds—The choice of seeds for the convenient production of asparagine is restricted by the availability of suitable species. Almost all of the information in the literature refers to seedlings of plants of the legume family. The classical work of Schulze was carried out chiefly with a number of species of *Lupinus* commonly grown in Europe, but few of these can be procured in this country at the present time. However, considerable stocks of a variety of *Lupinus albus* were found to be available in

Italian grocery stores in New England under the name "lupino bean" and, although this seed is probably imported, some of the present work was carried out with it. Another useful variety of this species was stocked by local wholesale seedsmen.¹

A number of varieties of vetches (*Vicia*) were studied but with no very satisfactory results. In many cases, germination was not rapid and uniform unless the seeds were treated with concentrated sulfuric acid for a period of 30 to 45 minutes.

Many varieties of beans (*Phaseolus*) doubtless produce moderately high concentrations of asparagine when the seedlings are etiolated and invite investigation from the present point of view, but studies with these species have not as yet been conducted. The soy bean (*Glycine hispida* var. Black Wilson),² however, was found to be suitable for the production of asparagine, although the yields were low as compared with those from white lupines.

Preparation of Etiolated Seedlings—The seedlings are grown in a closed cabinet provided with a drain for excess water and with an inlet for the mist of water droplets produced by a commercial air-humidifying device. Dimensions are immaterial; the cabinet used in the present experiments was constructed of metal and was 3 feet 8 inches wide, 2 feet 8 inches high, and the same in depth. It was installed in a dimly lit corner of a basement laboratory.

Trays were constructed with a wooden frame and a coarse wire mesh bottom and were given a coat of paraffin applied hot. Either two or three of them were supported over each other, suitably spaced, the lowest several inches above the bottom of the cabinet.

A weighed quantity of seed was spread evenly on the wire mesh to form a layer often two seeds thick, and the seeds were thoroughly sprayed with water from a hose fitted with a flat shower-bath type of spraying head at intervals of about 2 hours for the first few days, and thereafter several times a day throughout the period of growth. During the night, the humidity was maintained at as near 100 per cent as possible by connecting the humidifying device to the cabinet in such a way that a continuous slow current of air carrying a mist of fine water particles was supplied. For rapid growth, a temperature in the range 20–25° appears desirable but no precise control was attempted. There should be as little exposure to light as possible.

Under these conditions germination occurs within 2 or 3 days and growth

¹ F. H. Woodruff and Sons, Inc., Milford, Connecticut.

² Dr. J. R. Mohler of the Bureau of Animal Industry has informed us that a variety of soy bean known as Pekin has proved satisfactory for the production of asparagine in their laboratory. Doubtless many other varieties may also be used.

thereafter is rapid. The intense crowding of the seedlings is not important, since they depend entirely on their own resources for nutriment, only water and air being required. The frequent spraying of the seedlings washes the surfaces and prevents the accumulation of water in which bacterial or mold growth may have started. Complete and efficient drainage is necessary to secure this. As the roots develop, they should hang free from the lower side of the wire screen, although, as growth proceeds, those from the upper trays become entangled with the tops of the seedlings below them. Growth appears to proceed normally provided the roots are kept covered with a film of moisture.

TABLE I

Rate of Accumulation of Asparagine in Tissues of Seedlings of Lupinus albus Grown in Darkness and in Light

At the end of 30 days the etiolated plants had three small pale yellow leaves, invasion by microorganisms had begun, and the food stores were becoming exhausted. The plants in the light were dark green and healthy, and would probably have grown for a considerably longer period. The data are based on analyses of from twenty to thirty healthy plants from which the testas had been removed as soon as they could be slipped off of the swollen cotyledons.

Time	Plants in darkness		Plants in light	
	Dry weight per seedling	Asparagine hydrate per gm. dry tissue	Dry weight per seedling	Asparagine hydrate per gm. dry tissue
days	gm.	gm.	gm.	gm.
6	0.486	0.043	0.472	0.048
12	0.470	0.135	0.460	0.143
16	0.446	0.141	0.448	0.152
20	0.383	0.215	0.425	0.177
25	0.346	0.249	0.419	0.182
30	0.357	0.288	0.431	0.177

The length of the culture period depends on the species selected. With soy beans, there seems little advantage in prolonging culture beyond about 15 days, when the first leaflets are beginning to form (see Table III), but, with white lupines, asparagine accumulation continues as long as the plants can be preserved in health in darkness (25 to 30 days), and may reach one-quarter or more of the dry weight of the seedling tissue. Table I illustrates the approximately steady rate at which asparagine is synthesized in the dark in contrast to the falling off in rate after 20 days of culture in light. At this point, leaf tissue has become fully differentiated in light, and the effect of photosynthesis is shown by the slowing up of the rate of loss of dry weight of the plants as compared with the behavior in darkness where there is no compensation for the respiration loss. As a general rule,

it is necessary to terminate the culture when the roots begin to darken, and especially if any substantial number of them soften and become translucent through the invasion of microorganisms.

Extraction of Seedling Tissue—The procedure to be followed depends upon the equipment available. In this laboratory, the tissue, after being weighed, is ground in a meat grinder with the addition of dry filter paper clippings as necessary to enable the mill to force the somewhat slippery material through the coarse cutting plate. The mass is then put through the mill a second time with the use of a cutting plate with smaller holes. Preliminary freezing and thawing of the tissue may aid in this operation, or may make it possible to press out a considerable part of the juice before grinding is attempted. If a suitable mill is not available, the tissue may be cut up into short lengths and extracted with boiling water, as much extract as possible being decanted through a suitable screen. In either case, the mass is formed into square cakes, of convenient size folded in canvas press-cloth, which are pressed in a stack of two or three at a time between steel plates in a hydraulic press.³ The cakes are subsequently broken up, mixed with a moderate quantity of warm water (60°), and pressed again, and it is usually best to wash a second time if the highest possible yield is desired.

The extract is heated to 80–90° with steam in order to coagulate the protein in solution, and is allowed to cool. The reaction is adjusted, if necessary, to pH 6 or slightly below with acetic acid, and the precipitate is allowed to settle overnight. With soy bean extracts, it is necessary to acidify to a faint reaction to Congo red paper (pH 4.0) in order to secure flocculation of the precipitate.

The supernatant solution is decanted and filtered through a pulp filter covered with a thin layer of celite. The sludge obtained with soy bean extracts is mixed with a sufficient amount of filter paper clippings and celite to form a soft mass that can be formed into cakes and is pressed. The pressed residue should be washed at least once, and the extract so obtained is filtered together with the supernatant solution. The total volume of filtered extract is measured and an aliquot is removed for the determination of ammonia, glutamine and asparagine amide nitrogen, and soluble nitrogen (6).

Isolation of Asparagine—The extract is concentrated *in vacuo* in stills equipped with vapor coolers (7), or in other available rapid distillation apparatus. For control of frothing, the addition of a few drops of Turkey red oil has been found extremely useful, it being possible to operate at the

³ In the absence of a hydraulic press, the tissue may be wrapped in cheese-cloth and pressed by hand. If washed a sufficient number of times with hot water, a reasonably effective extraction may be thus secured, although at the expense of considerable labor, and with the accumulation of a large volume of solution.

full efficiency of the still with extracts that are otherwise unmanageable. Concentration is continued until the extract is reduced to a thick sirup. As a rough rule, the volume of the sirup, expressed in ml, should be about half the magnitude of the weight in gm. of the seeds from which the extract was derived. The sirup is transferred while still hot to centrifuge bottles, with the use of the minimum possible amount of hot water, and is then chilled in the refrigerator for 3 days, being occasionally thoroughly stirred to promote complete crystallization. Seeding with crystals of asparagine has been found unnecessary in most cases.

The crude asparagine is centrifuged off at low temperature. As a rule, a thick but clear supernatant solution is obtained which is carefully de-

TABLE II

Composition of Once Recrystallized Crops of Asparagine Obtained from Etiolated White Lupine and Soy Bean Seedlings

	Water of crystalliza- tion (theory 12.00)	Ash	Nitrogen (theory 18.66)	Purity
	per cent	per cent	per cent	per cent
Lupine 2	11.96	0.09	18.55	99.4
" 3	12.10	0.02	18.48	99.1
" 4	12.34	0.05	18.67	100.1
" 5, first crop	12.16	0.19	18.52	99.8
" 5, second "	12.16	0.35	18.35	98.7
" 6 and 7, first crop	12.07	0.01	18.17	97.4
" 6 " 7, second "	12.28	0.29	17.51	93.9
Soy bean 1, first crop	11.96	0.02	18.50	99.2
" " 1, second "	11.78	0.35	18.10	97.1
" " 2, first "	12.33	1.19	18.03	96.6
" " 2, second "	12.18	0.32	17.72	94.9

canted, together with as much of the intermediate layer of brown flocculent sludge as possible, the sirup being allowed to drain away thoroughly from the packed crystals. The crystals are then washed three times successively by centrifugation with about one-half their volume of ice water each time, the apparatus being kept ice-cold throughout the operations of stirring the crystals and draining of the wash fluid.

Recrystallization of Asparagine—The crude crystals are treated with about 5 times their weight of hot water and are heated on the steam bath at 80° until dissolved. A liberal quantity of norit together with about 5 gm. of celite per liter of solution is added, and the mixture is heated at 80° with stirring for a short period and is filtered through a pulp filter previously heated by being washed with boiling water. The residue is washed

once with a little hot water, and the filtrate, which should be perfectly clear although it is seldom completely free from color, is allowed to cool in the refrigerator overnight. It is best to stir occasionally during the crystallization to prevent the formation of a hard cake. The crystals are filtered on paper, washed with a little cold water, and then with dilute and finally with strong alcohol, and are dried in the air. Meanwhile the pulp filter and norit are thoroughly washed with boiling water, and these washings are combined with the filtrate and washings from the main crop of crystals and concentrated *in vacuo* for the crystallization of a second crop.

TABLE III

Data Obtained During Preparation of Etiolated Lupine and Soy Bean Seedlings, Extraction of These, and Preparation of Asparagine

	Weight of seeds	Age	Fresh weight	Extract volume	Asparagine hydrate by analysis	Asparagine N of soluble N	Yield on dry weight of seeds	Asparagine hydrate isolated	Yield of crystals on analytical result
	gm.	days	kg.	liters	gm.	per cent	per cent	gm.	per cent
Lupine 1.... .	55.2	26	0.668	6.2	8.46	69.7	15.3	8.19	96.8
" 2.... .	172	25	2.04	2.0	26.05	69.1	15.2	24.9	95.7
" 3....	350	27	3.85	13.6	50.3	66.1	14.4	50.2	99.8
" 4.... .	315	27	3.35	8.9	48.6	68.9	15.4	50.6	103.8
" 5.....	4195	28	47.12*	50	442.7		12.75		
" 5w†....				41	91.8		2.19	550.2	103
" 6.....	2000	30	17.43	39.7	131	32.5	6.5	341	99.8
" 7.... .	3000	26	23.1	54.2	214	36.1	7.13		
Soy bean 1..	3000	15	27.18	64	215.5	47.4	7.18	182.1	84.9
" " 2.....	3460	20	36.9	67.8	215.9	33.6	6.24	200.4	93.2

* Includes much testa tissue and is accordingly relatively high as compared with previous experiments in which testa tissue had been removed.

† Washings of the press residue obtained in extracting the tissue of Lupine 5. The high final yields in this and in the previous experiment are within the limits of accuracy of the method employed to analyze the extract.

Table II gives analytical results on several lots of asparagine obtained in this way and illustrates the degree of purity of the material so prepared. Completely pure material can be obtained by repeated crystallization under the same conditions.

In the present investigation, the main mother liquors and the mother liquors of recrystallization were worked over in order to obtain the maximum possible yield of asparagine. For practical purposes, especially with material derived from soy bean extracts, it is probably not worth while to attempt to secure another crude crop from the heavy sirup. For example, in the first experiment with soy bean extract, shown in Table III, the main

crop of recrystallized asparagine weighed 154.8 gm., while a further 18 gm. of less pure material were obtained from the mother liquor of recrystallization by evaporation and the use of alcohol. From the main sirupy mother liquor, after a laborious process that involved treatment with norit and celite, concentration, and precipitation with alcohol and subsequent purification of the asparagine obtained, only 9.2 gm. more were secured in reasonably pure form. Mother liquors from lupine extracts may repay evaporation for the crystallization of a second crude crop if the evaporation before the separation of the first crop was not carried far enough.

DISCUSSION

Table III shows data obtained during several isolations of asparagine from lupine seedlings and from soy bean seedlings. The first four experiments were preliminary small scale studies carried out in order to ascertain the best conditions for maximum yield. The isolation technique was varied from experiment to experiment and what appears to be the simplest procedure is described above. Several general principles were found to apply. The use of alcohol to promote the separation of asparagine should be avoided, since it likewise precipitates a flocculent impurity, probably of complex carbohydrate nature, that is difficult to remove later from the crystalline material. Furthermore, there is no advantage in the use of alcohol during the recrystallization provided the temperature of the aqueous solutions is kept low. In purifying crude asparagine by recrystallization, the temperature at which the solution is treated with norit should not be higher than 80°; otherwise a flocculent impurity may be precipitated which renders filtration slow and difficult. Filtration, especially of the sirupy concentrated solutions, is to be avoided in favor of centrifugation until the material has been freed from most of the impurities.

The high yields obtained from lupine seedlings as well as the reproducibility of the experiments indicate the special advantage of using this species. The extraordinarily high proportion of the soluble nitrogen in the form of asparagine in these extracts recalls the early work of Schulze (8), although it is to be noted that his even higher results refer to extracts prepared from the axial organs alone, whereas the present figures refer to the entire seedling and, in the large scale experiments, include a great deal of the testa tissue that Schulze invariably carefully removed. There seems little doubt that the axial organs of the present lupine seedlings yielded an extract that contained only a moderate proportion of nitrogenous material other than asparagine.

The experiment on Lupine 5 illustrates the desirability of thorough washing of the pressed tissue during the preliminary extraction. Experiments on Lupines 6 and 7, although unsatisfactory from the standpoint of aspara-

gine production, are most instructive. They represent seedling cultures carried out, the one on a painted concrete floor, the other on trays provided with impervious bottoms. In both cases drainage of the excess water was very poor and, as a result, infection of the rootlets and even of the hypocotyls was serious. The tissues from the two cultures were worked up together in order to ascertain the effect of this condition. The results of the analysis of the extracts show a greatly depressed yield of asparagine, and data not given indicate that considerable amounts of ammonia were present. The percentage recovery of such asparagine as survived in the two extracts was satisfactory, but considerable difficulty was experienced in obtaining pure products. Obviously this technique of preparing the etiolated seedlings is unsatisfactory.

The two experiments with soy beans show the marked difference of this species from lupines with respect to asparagine metabolism. The longer culture period in the second experiment did not result in an increase in asparagine; on the contrary, a significantly lower proportion of the soluble nitrogen was present in this form, and data not given indicated the presence of a much increased proportion of ammonia in the extract. Furthermore, in sharp contrast to the behavior of lupine seedlings, extracts of soy bean seedlings contain substantial quantities of glutamine. In both of these extracts, glutamine was present in an amount equal to about one-sixth that of asparagine.

In the present work, the use of basic lead acetate, invariably employed by Schulze to clarify the tissue extracts, has been avoided, and what is really a return to the early technique of Piria is advocated. However, there is little doubt that more satisfactory solutions are secured after treatment of the extract with this reagent. Crystallization of the asparagine then becomes relatively easier and purification of the crude crop is facilitated. On the other hand, a long filtration to remove the voluminous lead acetate precipitate and the subsequent washing of this have been avoided, as well as the removal of the excess lead as sulfide or sulfate, and the washing of this precipitate. The volumes of solution that must be evaporated if lead acetate is used become formidable. It is apparent from the present results that there is no necessary sacrifice in yield by the simplified procedure and that a satisfactorily pure product can be made without undue expense.

Through the courtesy of Professor Burkholder of Yale University, an examination for thiamine of several of the lots of asparagine obtained from lupines has been made. Professor Burkholder reports that none of the samples tested gave evidence for the presence of this vitamin although most samples of commercially obtained asparagine contain troublesome quantities when the material is used in culture media without further purification.

SUMMARY

Directions are given for the production of etiolated seedlings, either of lupines or of soy beans, and for the extraction of the tissues and isolation of asparagine. It has been found possible to obtain the asparagine in good yield by direct crystallization from the concentrated extract. Clarification with basic lead acetate according to the classical technique of Schulze is unnecessary. The final product may be purified to any desired degree by recrystallization from water.

BIBLIOGRAPHY

1. Long, E. R., and Seibert, F. B., *Am. Rev. Tuberc.*, **13**, 393 (1926).
2. Schulze, E., and Winterstein, E., in Abderhalden, E., *Handbuch der biochemischen Arbeitsmethoden*, Berlin, **2**, 510 (1910).
3. Murneck, A. E., *Plant Physiol.*, **10**, 447 (1935).
4. Vickery, H. B., and Schmidt, C. L. A., *Chem. Rev.*, **9**, 169 (1931). Vickery, H. B., Pucher, G. W., Wakeman, A. J., and Leavenworth, C. S., *Connecticut Agric. Exp. Stat., Bull.* 399 (1937).
5. Piria, R., *Ann. chim. et physiq.*, **22**, 160 (1848).
6. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., *Ind. and Eng. Chem., Anal. Ed.*, **7**, 152 (1935).
7. Vickery, H. B., and Pucher, G. W., *Ind. and Eng. Chem., Anal. Ed.*, **6**, 372 (1934).
8. Chibb, A. C., *Protein metabolism in the plant*, New Haven, 39 (1939).

RESPIRATORY EXCRETION OF SELENIUM STUDIED WITH THE RADIOACTIVE ISOTOPE*

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It is well known that the animal organism has the ability of converting both inorganic selenium and tellurium into volatile substances which are exhaled in the respiratory gases. The nature of the volatile tellurium compound has been shown to be a methylated telluride, while the contention of Hofmeister that the exhaled selenium compound is likewise a methyl derivative (1) lacks to date adequate experimental evidence (2). The metabolism of these volatile substances is not definitely known.

Schultz and Lewis (2) have reported quantitative studies on the excretion of volatile respired selenium after the administration of *sodium selenite*. They found after subcutaneous injection of sodium selenite (2.5 to 3.5 mg. of selenium per kilo) that 17 to 52 per cent of the injected selenium was excreted within 8 hours as a volatile compound in the respiratory gases. These results do not agree with our observations following the subcutaneous injection of subtoxic amounts of *sodium selenate* (radioselenium), in which 3 to 10 per cent of the original dose of selenium was exhaled within 24 hours.

The radioactive isotope of selenium as previously shown (3) affords an extremely sensitive analytical method for studying the metabolism of tracer amounts of this metal in the animal organism. In order to obtain information concerning the rate of formation and excretion of these volatile selenium compounds in the intact organism, time-excretion studies of exhaled selenium after the administration of *sodium selenate* have been made in rats with radioselenium as a tagged atom. It was found that the formation and excretion of volatile selenium compounds take place shortly after the administration of the inorganic selenium, and that the rate of excretion of these volatile substances through the lungs is more rapid during the first 3 hours than during any subsequent 3 hour period.

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EXPERIMENTAL

Young adult, male rats were injected subcutaneously with a single sub-toxic dose of sodium selenate (3 to 4 mg. of Se per kilo) containing radio-selenium. Analyses of respiratory selenium were made on twelve animals, on seven of which determinations were made at various intervals totaling up to 24 hours, and five single determinations for the entire 24 hour period on the other five.

A solution of 48 per cent hydrobromic acid and 5 per cent bromine was chosen as the absorbent for exhaled selenium, as it was shown by Dudley (4) that this solution collected volatile selenium such as hydrogen selenide, methyl selenide, ethyl selenide, and selenium dioxide very efficiently. It is quite possible that the volatile selenium exhaled by the animal is similar or identical to one or more of the compounds mentioned above.

The absorption apparatus consisted of a quart jar in which the experimental animal was placed. This was connected in series with two gas absorption chambers similar in principle to the Nichols chamber (5). Rubber surfaces were carefully waxed and glass connections tightly fitted with rubber sleeves. A steady stream of air was drawn through the system with a water pump.

To the hydrobromic acid and bromine solution containing the exhaled selenium, a small amount of non-radioselenium was added as the bromide which, in turn, acted as a carrier for the radioselenium. Enough distilled water was then added to reduce the concentrated hydrobromic acid to 25 to 30 per cent. The selenium was then precipitated by reducing the selenium bromide with sulfur dioxide followed by a small amount of hydroxylamine hydrochloride. The mixture was allowed to stand overnight. Selenium was then separated by centrifuging, dissolved in a small amount of HBr-Br_2 mixture, and made up to a suitable volume. An aliquot was taken for radioactive measurements. These were made as described elsewhere (6) on a Geiger-Müller counter of a scale of four.

As shown in Table I, quantitative recoveries of radioselenium were obtained when selenium was added as the bromide (SeBr_2) to an equivalent amount of absorbent solution and carried through all of the experimental steps outlined above.

Nearly all of the selenium in the experimental runs was found to be present in the first of the two absorption chambers. In two experiments in which a third absorption chamber contained concentrated H_2SO_4 instead of HBr-Br_2 , small amounts of radioselenium (an average of 3.6 per cent of the total) were found. It appears therefore that practically all the selenium exhaled by the experimental animal was absorbed in the HBr-Br_2 solution. The preparation of selenic acid and its sodium salt has been described (3).

TABLE I
Recovery Studies

Average net count represents the average count per minute (minus background) times a dilution factor. In Experiments 1, 2, and 5, a known amount of radioselenium equivalent to the amount in the corresponding control was added to a hydrobromic acid-bromine solution containing 10 mg. of non-radioselenium which acted as a carrier for radioselenium. The selenium was then precipitated and the radioactivity of the precipitate determined as described above. In Experiments 3 and 4, radioselenium as the bromide was added to the first of two absorption chambers which contained hydrobromic acid-bromine solution and the system aerated for 24 hours. Radioselenium was then isolated separately from the two chambers, and the radioactivity measured as before. Experiments 3a and 4a represent the counts obtained in the first absorption chamber, while Experiments 3b and 4b represent counts found in the second chamber. The small amounts of radioselenium found in the second absorption chamber were probably carried over from the first chamber by mechanical means.

Experiment No.	Average net count	Average deviation
Control (counts added)	17.4	1.40
1 (Counts found)	18.5	1.20
2 " "	19.0	1.00
3a " "	15.4	0.27
3b " "	3.8	1.20
4a " "	16.3	0.90
4b " "	1.7	0.60
	19.2	
	18.0	
Control (counts added)	7.0	
5 (Counts found)	6.7	1.10

DISCUSSION

The rate of respiratory excretion of selenium over a 24 hour period is shown in Fig. 1. It will be noted that 3 to 10 per cent of the original dose of selenium was exhaled in 24 hours. It is obvious that the initial rapid rise in the time-excretion curve followed by a leveling off indicates that during the first 6 hours after injection the rate of respiratory excretion of selenium is more rapid than during the following 18 hours.

The administered percentages of selenium exhaled in 24 hours for various time intervals during the 24 hour period are shown in Fig. 2. It was found that, during the first 3 hours, an average of 50 per cent of the total selenium exhaled in 24 hours was exhaled, followed by an additional 25 per cent (average) in the next 3 hours. It thus follows that approximately 75 per cent of the total amount of selenium exhaled in 24 hours was excreted during the first 6 hours.

A careful survey of the literature reveals very little information concerning the metabolism (site and mode of formation) of the volatile selenium

compound or compounds that are readily produced in the animal organism following the administration of inorganic selenium. It is of interest in this connection to note, as observed in previous distribution studies (3), that peak concentrations in liver and lung were reached at 2 hours and $\frac{1}{2}$ hour respectively, and that these peak concentrations both in liver, which was the greatest of any tissue examined, and lung occur at a time when the rate of respiratory excretion of volatile selenium is at a maximum. It may well be that small amounts of selenium are mobilized in the liver more than in any other tissue for detoxication purposes, inasmuch as it is commonly stated that many detoxication processes do take place in the liver. Furthermore, the garlic odor characteristic of volatile selenium can be unmistakably de-

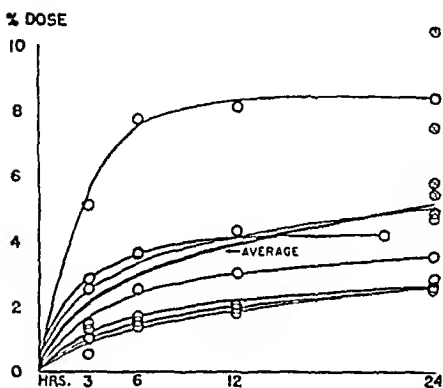


FIG. 1

FIG. 1. The rate of respiratory excretion of selenium after subcutaneous injection of sodium selenate. \circ represents a single 24 hour determination.

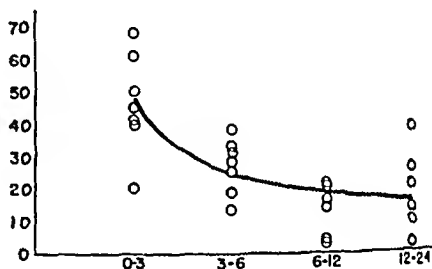


FIG. 2

FIG. 2. Per cent of total selenium exhaled (ordinate) at various time intervals measured in hours (abscissa) after the subcutaneous injection of sodium selenate.

tected in minced liver 2 hours after the injection of sodium selenate. It seems likely, in view of the foregoing, that the greater part of the administered inorganic selenium that is excreted through the respiratory tract is first mobilized in the liver where reduction and possible methylation take place. Then the formed volatile selenium compounds diffuse into the blood and are carried to the lungs where they are eliminated from the organ in the respiratory gases. The possibility that volatile selenium compounds may be formed in the kidney, gastrointestinal tract, or elsewhere, of course cannot be entirely eliminated.

Schultz and Lewis (2) have reported a quantitative excretion of volatile selenium, using a colorimetric method for the determination of selenium and concentrated H_2SO_4 as the absorbent for volatile respiratory gases. These

workers found that after subcutaneous injection of *sodium selenite*, 17 to 52 per cent of the administered selenium was excreted by way of the lungs within 8 hours. Respiratory excretion values presented here after administration of *sodium selenate* are 3 to 10 per cent of the original dose in 24 hours.

On the basis of weight, *sodium selenite* has been shown by Franke and Moxon (7) to be more toxic to the rat when administered intraperitoneally than sodium selenate.¹ Of interest is the fact that selenite, the more toxic of the two inorganic compounds, is excreted as a volatile substance through the lungs in greater amounts than the less toxic selenate. It would appear that *sodium selenite* by virtue of its intrinsic properties (more reduced state, less stability, and greater toxicity) is more readily converted into volatile substances and is eliminated by way of the respiratory tract not only at a faster rate, but in greater amounts than is the more stable sodium selenate.

A report by Westfall and Smith (9) affords evidence to support the contention that sodium selenate follows different metabolic routes than does sodium selenite. After intravenous administration of sodium selenate, selenium appeared in fractions similar to those which occurred when sodium selenate was added to normal urine. 85 per cent of the selenium was found in an inorganic form, presumably selenate. However, when sodium selenite was administered in the same manner, only 15 per cent of the excreted selenium appeared in the same fractions as when added *in vitro*, while 80 per cent appeared in the ethereal and neutral sulfur fraction. These results were interpreted as evidence that there was a rapid "working over" of sodium selenite. The fact that 45 and 30 per cent of the injected dose was excreted in the urine in 24 hours, for sodium selenate and sodium selenite respectively (9), further substantiates the contention that selenate and selenite follow quantitatively different metabolic routes in the animal organism.

It is obvious that there is at present a need for the identification of the volatile substances which appear in the respiratory gases after the administration of inorganic selenium. With the discovery of the nature of these substances, progress will be made toward a more complete understanding of this particular phase of selenium metabolism which is very obscure at the present.

SUMMARY

With radioactive selenium as a tagged atom, time-excretion studies of exhaled selenium were made on rats after subcutaneous injection of sodium

¹ For a discussion concerning the relative toxicity of the various selenium compounds see Painter (8).

selenate. It was found that 3 to 10 per cent of the original dose was exhaled within 24 hours, and approximately 75 per cent of this amount was excreted during the first 6 hours.

The relation of these findings to selenium metabolism and detoxication is discussed.

The author wishes to express his appreciation to Dr. Robert R. Sealock and Dr. William F. Bale for their helpful advice and criticism throughout this investigation. The author would also like to thank Dr. J. P. Lambooy for his aid in the construction of the absorption chambers.

BIBLIOGRAPHY

1. Hofmeister, F., *Arch. exp. Path. u. Pharmacol.*, **33**, 198 (1893-94).
2. Schultz, J., and Lewis, H. B., *J. Biol. Chem.*, **133**, 199 (1940).
3. McConnell, K. P., *J. Biol. Chem.*, **141**, 427 (1941).
4. Dudley, H. C., *Am. J. Hyg.*, **24**, 227 (1936).
5. Nichols, M. S., *Pub. Health Rep., U. S. P. H. S.*, **53**, 538 (1938).
6. Bale, W. F., Haven, F., and LeFevre, M. L., *Rev. Scient. Instruments*, **10**, 193 (1939).
7. Franke, K. W., and Moxon, A. L., *J. Pharmacol. and Exp. Therap.*, **58**, 454 (1936).
8. Painter, E. P., *Chem. Rev.*, **28**, 183 (1941).
9. Westfall, B. B., and Smith, M. I., *J. Pharmacol. and Exp. Therap.*, **72**, 245 (1941).

SYNTHESIS OF DIMETHYL ETHERS OF THE TWO ENANTIOMORPHIC α -BUTYRINS AND THEIR HYDROLYSIS BY LIPASES

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The first to investigate stereochemical selectivity of esterases was Dakin (1) who, after treating racemic esters of mandelic acid with liver enzyme, observed optical activity of the products of hydrolysis. Mayer (2), Neuberg and Rosenberg (3), and later Abderhalden and Weil (4) made similar observations with glycerides as substrates. The early literature on the subject is comprehensively collected by Rona and Ammon (5) and recently by Bamann and Ammon (6).

Recent investigations indicate that the bulk of the natural fats is composed of mixed triglycerides (7). This type of substitution produces an asymmetric β -carbon atom. The resulting enantiomorphism should have some significance in biological processes involving natural fats. As materials for investigation, pure optically active glycerides are needed. In a recently published review (8) we have pointed out that the possibility of isolating such individual glycerides from natural sources seems remote at the present moment. The alternative approach to obtaining enantiomorphs of known constitution and configuration is by synthesis. Methods of synthesis developed in this laboratory during the past several years (8) have now made available pure optically active glycerides for enzymatic studies.

The simplest substrates for enzymatic investigation are the optically active α -monosubstituted glycerol derivatives. A considerable difference in the rate of hydrolysis of the individual α -glycerophosphates by phosphatases has been reported previously (9). Unlike the α -glycerophosphates, α -monoglycerides of aliphatic acids, however, show a great tendency to undergo acyl migration (10). Consequently, we have employed substituted α -monobutyryns in which acyl migration was made impossible, either by acetonation of the adjacent hydroxyls (the resulting acetone compound being stable against alkali but sensitive to acid), or by methylation (yielding ethers stable towards both alkali and acid). The present paper reports the results obtained with the methylated α -monobutyryns.

To prepare the two enantiomorphic dimethyl ethers of *d*(+)- α -butyryl glycerol (I) and *l*(-)- α -butyryl glycerol (II), considerable preparative work had to be done which is elucidated by Table I.

The method for the preparation of compounds (I) and (II) is in principle the same as that for the preparation of the corresponding optically active

TABLE I

Preparation of Enantiomorphous Dimethyl Ethers of d(+)- α -Butyryl Glycerol and l(-)- α -Butyryl Glycerol

575 gm.* mesquite gum	
↓	
164 gm. l-arabinose (11)	
↓	
47.5 gm. l-mannonolactone (12)	
↓ 65%	
33.5 gm. l-mannitol (13)	
↓ 70%	
51.1 gm. triacetone l-mannitol (13)	d-Mannitol
↓ 47.4%	↓
17.8 gm. 3,4-acetone l-mannitol†	Triacetone d-mannitol (14)
↓ 54.7%	↓
12.7 gm. tetramethyl-3,4-acetone l-mannitol†	3,4-Acetone d-mannitol (15, 16)
↓ 85%	↓
9.2 gm. 1,2,5,6-tetramethyl l-mannitol†	Tetramethyl-3,4-acetone d-mannitol (15)
↓ 57%	↓
5.15 gm. dimethyl-l-glyceraldehyde‡	1,2,5,6-Tetramethyl d-mannitol (15)
↓ 64.4%	↓
3.6 gm. l- α,β -dimethyl glycerol	Dimethyl-d-glyceraldehyde‡
↓ 79.2%	↓
4.52 gm. dimethyl ether of d(+)- α -butyryl glycerol (I)	d- α,β -Dimethyl glycerol
$\text{CH}_2 \cdot \text{O} \cdot \text{CO} - \text{CH}_2 - \text{CH}_2 - \text{CH}_3$	↓
	Dimethyl ether of l(-)- α -butyryl glycerol (II)
$\text{CH}_3 \cdot \text{O} \cdot \text{CH}$	$\text{H}_2\text{C} \cdot \text{O} \cdot \text{CO} - \text{CH}_2 - \text{CH}_2 - \text{CH}_3$
$\text{CH}_2 \cdot \text{O} \cdot \text{CH}_3$	$\text{HC} \cdot \text{O} \cdot \text{CH}_3$
(I)	
	$\text{H}_2\text{C} \cdot \text{O} \cdot \text{CH}_3$
	(II)

* The quantities for obtaining (II) from the commercially available d-mannitol are not mentioned because they are just the same as those given in the corresponding steps to prepare (I).

† A new substance made according to the prescription of Irvine and Paterson for the enantiomorph (15).

‡ Usually the dimethyl-d-glyceraldehyde and dimethyl-l-glyceraldehyde were not isolated as the pure substances, but the crude products obtained were reduced immediately to the corresponding dimethyl glycerols.

acetone glycerols. The optical classification of (I) and (II) was established according to the same principles as were used in elucidating the classifica-

tion of the acetone compounds (17) and will be more readily understood if the formulas in our previous papers (13, 18) are consulted. In using these formulas one only needs to substitute four methyl groups for the two isopropylidene groups.

The descriptions in the experimental part are given briefly, since the techniques employed follow the general methods described in our previous publications.

The action of the serum and liver lipases from rabbit, guinea pig, mouse, and rat on substrates (I) and (II) was investigated. The progress of the

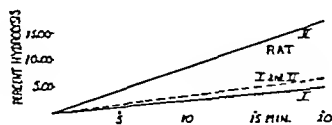


FIG. 1

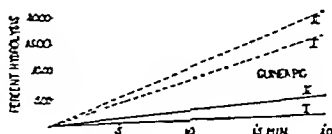


FIG. 2

FIG. 1. The action of serum and liver lipases of the rat on substrates (I) and (II). Curve I represents graphically the rate of hydrolysis of the dimethyl ether of *d*(+)- α -butyryl glycerol and Curve II that of the dimethyl ether of *l*(-)- α -butyryl glycerol. The solid lines indicate hydrolysis by serum lipase; the dotted lines, hydrolysis by liver lipase.

FIG. 2. The action of serum and liver lipases of the guinea pig on substrates (I) and (II). The curves have the same significance as those in Fig. 1.

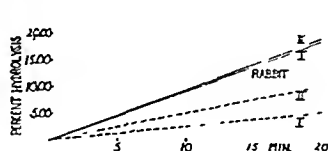


FIG. 3

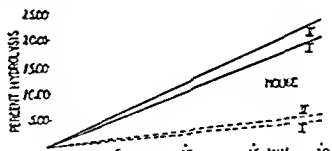


FIG. 4

FIG. 3. The action of serum and liver lipases of the rabbit on substrates (I) and (II). The curves have the same significance as those in Fig. 1.

FIG. 4. The action of serum and liver lipases of the mouse on substrates (I) and (II). The curves have the same significance as those in Fig. 1.

enzymatic hydrolysis was followed by continuous titration at a definite pH (19). The results are given in Figs. 1 to 4.

Under the conditions specified in the experimental part, considerable differences in the speed of hydrolysis of compounds (I) and (II) have been found in several instances. It has to be emphasized that these differences usually vary with changing conditions (*e.g.*, substrate concentrations). Experiments carried out in this laboratory with the enantiomorphic acetone α -butyrins showed also significantly large differences in the speed of hydrolysis. Results of these experiments will be reported later.

The methylated glycerides do not constitute a material likely to occur in nature and were chosen only to avoid acyl migration. No further experiments were carried out, as we felt that the results obtained were sufficient to strengthen our concept that enantiomorphic forms of glycerides may behave differently towards enzymes.

EXPERIMENTAL

Preparation of Substrates

3,4-Acetone l-Mannitol—This substance was prepared by acid hydrolysis of triacetone *l*-mannitol according to the method of Irvine and Paterson (15) for the preparation of 3,4-acetone *d*-mannitol, but the modification of Fischer and Appel (16) was used. 51.1 gm. of triacetone *l*-mannitol yielded 17.8 gm. (47 per cent) of 3,4-acetone *l*-mannitol, m.p. 85–86.5°.

$C_9H_{16}O_6$ (222.14). Calculated. C 48.65, H 8.20

Found. " 48.84, " 8.24

Optical Rotation—In water, 1 dm. tube, $c = 4.23$, $\alpha_D = -1.25^\circ$, $[\alpha]_D = -29.6^\circ$.

Tetramethyl-3,4-acetone l-Mannitol—This was prepared according to the method of Irvine and Paterson (15) for its enantiomorphic form, by methylation of 3,4-acetone *l*-mannitol with silver oxide and methyl iodide. 17.8 gm. of 3,4-acetone *l*-mannitol yielded 12.2 gm. of tetramethyl-3,4-acetone *l*-mannitol (54.7 per cent), b.p. (9 to 10 mm.) 132–134°.

$C_{13}H_{26}O_6$ (278.2). Calculated. C 56.12, H 9.40

Found. " 55.90, " 9.39

Optical Rotation—In water, 1 dm. tube, $c = 3.33$, $\alpha_D = -1.30^\circ$, $[\alpha]_D^{20} = -39.0^\circ$.

1,2,5,6-Tetramethyl l-Mannitol—This compound was prepared by acid hydrolysis according to the method of Irvine and Paterson (15) for the preparation of its enantiomorph. 12.7 gm. of tetramethyl-3,4-acetone *l*-mannitol yielded 9.2 gm. of tetramethyl *l*-mannitol (85 per cent), b.p. (9 to 10 mm.) 152–157°.

$C_{10}H_{22}O_6$ (238.2). Calculated. C 50.42, H 9.30

Found. " 50.24, " 9.55

Optical Rotation—In water, 1 dm. tube, $c = 3.94$, $\alpha_D = +0.52^\circ$, $[\alpha]_D = +13.2^\circ$.

l- α,β -Dimethyl Glycerol. Preparation of Dimethyl-l-glyceraldehyde—To a solution of 9.2 gm. of 1,2,5,6-tetramethyl *l*-mannitol in 200 cc. of dry benzene were added 17.2 gm. of lead tetraacetate and the solution was stirred for half an hour at room temperature. After addition of 50 cc. of dry ether, the lead diacetate was centrifuged off and the aldehyde solution was carefully concentrated as completely as possible with the use of an efficient fractionating column. The residue was redissolved in 40 cc. of ether and the precipitate of lead salts was filtered off. The ethereal solution was concentrated by slowly running it into a small distilling flask

immersed in a bath of 40–60°. The remaining impure dimethylglyceraldehyde distilled at 37–42° under 8 to 10 mm. of Hg pressure (bath temperature 60–80°), yielding 5.15 gm. (57 per cent) of dimethyl-*l*-glyceraldehyde.

The reduction of dimethyl-*l*-glyceraldehyde was carried out immediately after the substance was obtained.

Reduction to *l*- α , β -Dimethyl Glycerol—To a solution of 5.15 gm. of freshly prepared dimethyl-*l*-glyceraldehyde in 40 cc. of ethyl acetate saturated with water were added 3 gm. of Raney's nickel catalyst. The substance was reduced at room temperature in a rotating autoclave with a glass container at an initial pressure of 80 atmospheres of hydrogen. After addition of a further 3 gm. of nickel catalyst, the reduction was continued for 2 days more. When the reduction was complete, the catalyst was centrifuged off and washed several times with ethyl acetate. The combined filtrates were dried with potassium carbonate and concentrated with the aid of a fractionating column. The residue was distilled in the vacuum of a water pump. Yield, 3.6 gm. (68.7 per cent) of pure *l*- α , β -dimethyl glycerol,¹ b.p. (7 mm. of Hg) 65–66° (bath 75–85°), $n_D^{21} = 1.4232$.

$C_5H_{12}O_4$ (120). Calculated. C 50.0, H 10.08

Found. " 50.25, " 10.24

Optical Rotation—(a) In homogeneous substance, 1 dm. tube, $d_D^{22} = 1.023$, $\alpha_D = +4.90^\circ$, $[\alpha]_D = +4.8^\circ$. (b) In water, 1 dm. tube, $c = 13.65$, $\alpha_D = -0.92^\circ$, $[\alpha]_D = -6.7^\circ$.

Dimethyl Ether of *d*(+)- α -(*n*-Butyryl) Glycerol (I)—To a mixture of 3.6 gm. of *l*-dimethyl glycerol and 3.9 gm. of dry quinoline, 3.18 gm. of *n*-butyryl chloride were added little by little, with occasional cooling, and the preparation, which soon solidified, was kept for 24 hours at room temperature. After dilution with 50 cc. of ether, the solution was washed successively three times with 5 cc. of 5 N sulfuric acid (saturated with sodium sulfate), twice with 4 cc. of saturated sodium sulfate solution, and once with 4 cc. of sodium bicarbonate solution. The ethereal solution was dried with sodium sulfate and concentrated. By fractional distillation of the residue in the vacuum of a water pump, 4.52 gm. (79.2 per cent) of analytically pure butyryl ester were obtained, b.p. (8 mm.) 94.5–95.5°, $n_D^{24.5} = 1.4198$, $n_D^{22} = 1.4208$. The substance is readily soluble in water.

$C_9H_{18}O_4$ (190). Calculated. C 56.8, H 9.5

Found. " 56.8, " 9.5

Optical Rotation—In homogeneous substance, 0.5 dm. tube, $d_D^{22} = 0.988$, $\alpha_D = +2.91^\circ$, $[\alpha]_D = +5.9^\circ$.

¹ The glycerol was free from traces of aldehyde, as no reduction of ammoniacal silver nitrate solution could be observed.

² A similar reversion of rotation was observed for the acetone glycerols ((13) and (18) p. 469).

d- α,β -Dimethyl Glycerol. Preparation of Dimethyl-*d*-glyceraldehyde—The dimethyl-*d*-glyceraldehyde was prepared as described above for its isomer. 11.4 gm. of 1,2,5,6-tetramethyl *d*-mannitol yielded 6.2 gm. of dimethyl-*d*-glyceraldehyde (twice fractionated), b.p. (8 mm.) 38.5–39.0° (bath 45–50°).

$C_6H_{10}O_3$ (118). Calculated. C 50.8, H 8.5
Found. " 50.65, " 8.58

Optical Rotation—In benzene, $c = 10.71$, 1 dm. tube, $\alpha_D = +10.5^\circ$, $[\alpha]_D = +98.0^\circ$.

Reduction to *d*- α,β -Dimethyl Glycerol—Prepared according to the direction for its isomer given above, 6.0 gm. of dimethyl-*d*-glyceraldehyde yielded 4.52 gm. of *d*-dimethyl glycerol (74 per cent), b.p. (8 mm.) 67.2–67.4°, $n_D^{25} = 1.4218$.

$C_6H_{12}O_3$ (120). Calculated. C 50.0, H 10.08
Found. " 50.19, " 10.08

Optical Rotation—(a) In homogeneous substance, 1 dm. tube, $d_4^{25} = 1.028$, $\alpha_D = -4.88^\circ$, $[\alpha]_D = -4.75^\circ$. (b) In water, $c = 14.15$, 1 dm. tube, $\alpha_D = +0.96^\circ$, $[\alpha]_D = +6.8^\circ$.

Dimethyl Ether of *l*(-)- α -(*n*-Butyryl) Glycerol—This substance was prepared by the method described above for its isomer; 4.52 gm. of *d*-dimethyl glycerol yielded 5.88 gm. (82 per cent) of dimethyl ether of *l*(-)-butyryl glycerol, b.p. (8 mm.) 93.5–94°, $n_D^{25.5} = 1.4202$.

$C_8H_{18}O_4$ (190). Calculated. C 56.80, H 9.55
Found. " 56.71, " 9.34

Optical Rotation—In homogeneous substance, 1 dm. tube, $d_4^{25} = 0.988$, $\alpha_D = -5.96^\circ$, $[\alpha]_D = -6.0^\circ$.

Enzymatic Investigations

Substrate Solutions—All enzymatic experiments were carried out with 0.04 M aqueous solutions³ prepared by dissolving 0.760 gm. of the dimethyl ethers of *l*(-)- α -butyryl glycerol and *d*(+)- α -butyryl glycerol in distilled water and making up to 100.0 cc. The substrate solutions remained unaltered for several days if kept in an ice box. The hydrolysis of butyric esters in the absence of enzyme and at a pH of 7.2 during the experiment is so slight that it is not necessary to subtract a blank value.

Source of Enzymes—Blood serum and aqueous liver extract from guinea pigs, mice, rabbits, and rats were used. The sera were prepared in the usual way, the guinea pig serum being diluted with 1.5 volumes of water before use.

The liver extracts were made as follows: Immediately after the animal was killed by means of a blow on the head and then cutting the jugular

³ The difficulties encountered when emulsions were used were avoided, because the substrates are sufficiently soluble in water to give solutions.

vein, the liver was removed and ground with 5 times its weight of water. The suspension was allowed to extract for 15 hours in the refrigerator. It was then centrifuged. The extracts were so active that dilutions of 10 parts of water for guinea pig liver extract, 15 parts of water for mouse liver extract, 30 parts of water for rabbit liver extract, and 10 parts of water for rat liver extract had to be employed to keep the hydrolysis running at a suitable rate.

Enzymatic Hydrolysis—The progress of the hydrolysis of the enantiomorph butyric acid esters, (I) and (II), by the various preparations of lipases was followed by the method of continuous titration (19),⁴ at pH 7.2 in a bath of constant temperature (37°). After 10.0 cc. of 0.04 M substrate solution, colored with 4 drops of an aqueous solution of phenol red, were brought to 37°, the experiments were started by adding 0.2 cc. of serum⁵ or liver extract. Immediately on addition of the enzyme to the substrate solution, 0.01 N sodium hydroxide was dropped into the reaction mixture at a speed which maintained the color of the mixture, the same as that of a buffered comparison solution (14.0 cc. of M/15 secondary sodium phosphate plus 6.0 cc. of M/15 primary potassium phosphate plus 6 drops of a phenol red solution) at pH 7.2. Readings were taken every 2 minutes for 20 minutes. During this period the line showing the rate of hydrolysis remains straight (*i.e.*, the initial rate was maintained).

The racemic dimethyl ether of α -butyryl glycerol was not obtained in the same state of purity as that of enantiomorphous forms, since it had to be synthesized in a different way. For this reason the hydrolysis curves of the racemic compound were only approximately equidistant from those of its enantiomorphous forms. Although they served the purpose of confirming the other curves, there seemed no object in including them in Figs. 1 to 4.

SUMMARY

The dimethyl ethers of *d*- and *l*- α -butyryl have been synthesized from *d*- and *l*-mannitol.

Serum lipase of rats and guinea pigs and liver lipase of rabbits were found to hydrolyze the enantiomorphs with a considerable difference in velocity.

BIBLIOGRAPHY

1. Dakin, H. D., *J. Physiol.*, 30, 253 (1904); 32, 199 (1905); *Proc. Chem. Soc.*, 19, 161 (1903).
2. Mayer, P., *Biochem. Z.*, 1, 39 (1906).

⁴ Experiments carried out in the Warburg-Barcroft apparatus confirmed the results arrived at by continuous titration.

⁵ Except in the case of rabbit serum, when 1.0 cc. was added.

3. Neuberg, C., and Rosenberg, E., *Biochem. Z.*, **7**, 191 (1907).
4. Abderhalden, E., and Weil, A., *Fermentforschung*, **4**, 76 (1920); *Chcm. Zentr.*, **III**, 643 (1920).
5. Rona, P., and Ammon, R., in Nord, F. F., and Weidenhagen, R., *Ergebnisse der Enzymforschung*, Leipzig, **2**, 50-73 (1933). Ammon, R., in Nord, F. F., and Weidenhagen, R., *Handbueh der Enzymologie*, Leipzig, 370-380 (1940).
6. Bamann, E., and Ammon, R., in Bamann, E., and Myrbäck, K., *Die Methoden der Fermentforschung*, Leipzig, 1704-1716 (1940).
7. Cf. the literature in Daubert, B. F., and King, C. G., *Chcm. Rev.*, **29**, 270 (1941).
8. Fiseher, H. O. L., and Baer, E., *Chem. Rev.*, **29**, 287 (1941).
9. Baer, E., and Fiseher, H. O. L., *J. Biol. Chcm.*, **135**, 321 (1940).
10. Fiseher, E., *Bcr. chcm. Ges.*, **53**, 1621 (1920). Jackson, D. T., and King, C. G., *J. Am. Chcm. Soc.*, **55**, 678 (1933). Hibbert, H., and Greig, M. E., *Canad. J. Research*, **4**, 254 (1931).
11. Anderson, E., and Sands, L., in Gilman, H., *et al.*, *Organic syntheses*, New York, 2nd edition, coll. **1**, 67-70 (1941); *Ind. and Eng. Chcm.*, **17**, 1257 (1925); *J. Am. Chem. Soc.*, **48**, 3172 (1926).
12. Kiliani, H., *Ber. chem. Ges.*, **55**, 100 (1922); **58**, 2349 (1925).
13. Baer, E., and Fiseher, H. O. L., *J. Am. Chem. Soc.*, **61**, 763 (1939).
14. Fiseher, E., *Ber. chem. Ges.*, **28**, 1168 (1895). Fiseher, H. O. L., and Taube, C., *Ber. chem. Ges.*, **60**, 487 (1927). See also ((13) p. 764).
15. Irvine, J. C., and Paterson, B. M., *J. Chem. Soc.*, **105**, 907 (1914).
16. Fischer, H. O. L., and Appel, H., *Hclv. chim. acta*, **17**, 1576 (1934).
17. Baer, E., and Fiseher, H. O. L., *J. Biol. Chem.*, **128**, 478 (1939); *Chem. Rev.*, **29**, 299 (1941).
18. Baer, E., and Fiseher, H. O. L., *J. Biol. Chem.*, **128**, 464 (1939).
19. Hall, G. E., and Lucas, C. C., *J. Pharmacol. and Exp. Therap.*, **59**, 34 (1937).

CARBOXYLASES OF ANIMAL TISSUES

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The mechanisms which have been suggested for the metabolism of pyruvic acid in animal tissues have been based mainly on experiments with tissue slices or breis. The purpose of the present investigation was to attempt to facilitate the study of pyruvate metabolism by the preparation from animal tissues of a cell-free enzyme which could decarboxylate pyruvic acid. As a working hypothesis, the assumption was made that the enzyme sought was a diphosphothiamine-protein compound (1); and pig heart was chosen as the starting material because of its high thiamine content (2). By thus using bound diphosphothiamine as a tracer, we were able to obtain an enzyme preparation which decarboxylated α -keto acids anaerobically (3). In the presence of this enzyme, pyruvic acid formed acetoin and CO_2 , α -ketobutyric acid formed propionin and CO_2 , and α -ketoglutaric acid formed succinic semialdehyde and CO_2 . Added acetaldehyde and propionaldehyde reacted with both pyruvic acid and α -ketobutyric acid in the presence of the enzyme to form various acyloins in which part of the molecule was derived from the aldehyde and part from the α -keto acid. The enzyme preparation also acted slowly upon acetaldehyde alone to give acetoin. All these enzyme activities were found to depend upon the presence of diphosphothiamine.

Methods

In the initial search for the enzyme, no attempt was made to follow enzyme activity. Various extractions and fractionations were tried with the object of separating a diphosphothiamine-protein complex. Bound diphosphothiamine was determined as the increase in free diphosphothiamine after denaturation of the protein by boiling. The protein moiety of yeast carboxylase, supplemented with magnesium ions and pyruvate, served as an assay system for free diphosphothiamine (4). Experiments on minced pig heart muscle indicated that most of the bound diphosphothiamine was associated with the insoluble protein fraction. By application of one of the methods previously described for concentrating the

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so called insoluble enzymes of heart muscle (5), we were able to obtain a preparation which catalyzed the anaerobic decarboxylation of pyruvic acid.

Unless otherwise specified, the following procedure was employed for the preparation of the enzyme for further study. Fresh pig heart muscle was minced twice in a coarse meat grinder and washed five times with 10 volumes of water. 80 gm. of the pressed out mince were mixed in a Waring blender with 40 cc. of 0.5 M β -glycerophosphate buffer, pH 6.0, and 240 gm. of crushed ice. After 10 minutes of homogenizing, the suspension was centrifuged. The cloudy supernatant fluid was decanted and mixed with 0.5 volume of crushed ice, and 10 per cent acetic acid was added cautiously to pH 4.6. The precipitate was rapidly centrifuged in the cold, and evenly resuspended in a mixture of 10 cc. of 0.5 M phosphate buffer, pH 6.0, and 2 cc. of 0.5 M NaHCO_3 . The final volume was about 20 cc. The final pH was 6.0.

All tests on the enzyme were conducted at 38°. CO_2 evolution was measured in Warburg vessels in an atmosphere of 95 per cent N_2 -5 per cent CO_2 , or 95 per cent O_2 -5 per cent CO_2 , or in air. When O_2 was present, controls to measure O_2 consumption were run with KOH in the center cup. The values for O_2 consumption were usually very small or negative. In general, the gas used had no effect on the reactions, which were all found to be completely anaerobic.

Bisulfite-binding titrations were run according to the method of Clift and Cook (6). Acetylmethylcarbinol was determined as nickel dimethylglyoxime according to Stahley and Werkman (7). In the experiment cited in Table II, pyruvate was determined by measuring the CO_2 liberated by $\text{Ce}(\text{SO}_4)_2$ oxidation (8). Acetaldehyde was determined by aeration of a trichloroacetic acid filtrate for 90 minutes at 38°, trapping the acetaldehyde in bisulfite solution, and titrating the bound bisulfite. Propionaldhyde was measured similarly except that the aeration was continued for 2 hours.

The α -ketoglutaric acid was prepared according to Neuberg and Ringer (9), oxalacetic acid according to Wohl and Oesterlin (10), α -ketocaproic acid according to Schoenheimer and Ratner (11), and phenylpyruvic acid according to Shemin and Herbst (12). Pyruvate was prepared according to Wendel (13).

Results

To obtain the maximum rate of decarboxylation of the substrates, the following components were necessary: (a) enzyme, (b) magnesium or manganese ions, and (c) diphosphothiamine. These facts are demonstrated in Table I in which the CO_2 evolved in 1 hour was measured with all components present and with one component left out. In the studies of the various reactions, the enzyme was therefore routinely supplemented with

Mg⁺⁺ and diphosphothiaminc. At the same pH, the enzyme system had greater activity in phosphate buffer than in citrate buffer (see below).

Decarboxylation of α -Ketoglutaric Acid—When the enzyme system was allowed to react with a limited quantity of α -ketoglutaric acid, approximately 1 molecule of CO₂ was liberated for each molecule of substrate added (Fig. 1). The original bisulfite titration remained virtually unchanged during the course of the reaction. For example, in three experiments 86, 90, and 101 per cent of the original bisulfite titration were obtained after the CO₂ evolution had proceeded to completion. The reaction product reduced methylene blue in the presence of xanthine aldehyde oxidase of milk. It was identified as succinic semialdehyde (formylpropionic acid) by isolation of the 2,4-dinitrophenylhydrazone derivative.

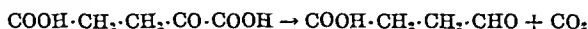
TABLE I

Components of Catalytic Systems for Decarboxylation of Pyruvic and α -Ketoglutaric Acids

The complete system contained 2 cc. of enzyme, 0.1 cc. of 0.5 per cent MgSO₄, 0.2 cc. of 0.1 per cent diphosphothiamine, and 0.2 cc. of 1 M substrate. The total volume of the fluid in the manometer vessels was made up to 3.0 cc. with water. The experiment was carried out in 95 per cent N₂, 5 per cent CO₂.

	CO ₂ in 60 min.	
	Pyruvic acid	α -Ketoglutaric acid
Complete system	230	282
Without diphosphothiamine	52	64
" magnesium	110	98
" substrate	0	0
With boiled enzyme	0	0

The decarboxylation of α -ketoglutaric acid proceeds, therefore, according to the equation



Decarboxylation of Pyruvic Acid—When pyruvate was added to the enzyme, and the reaction was allowed to go to completion, approximately 1 molecule of CO₂ was formed for each molecule of pyruvate added, provided the initial concentration of pyruvate was not too high (Fig. 2). No acetaldehyde could be detected. The reaction product did not reduce methylene blue in the presence of xanthine oxidase. The bisulfite titration gave irregular values because of the rapid drift in the first iodine end-point. The reaction product was identified as acetoin (acetylmethylcarbinol; 3-hydroxy-2-butanone) on the basis of the following evidence: (a) a positive

reaction in the Voges-Proskauer test with creatine and alkali (14), (b) the formation of nickel dimethylglyoxime after oxidation with FeCl_3 , and (c) the isolation of the 2,4-dinitrophenylosazone of acetoin.

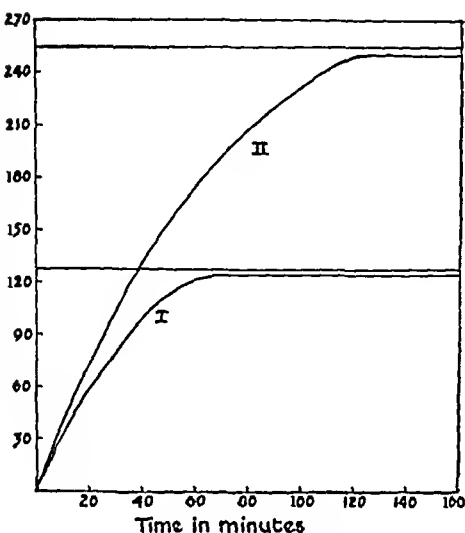


FIG. 1

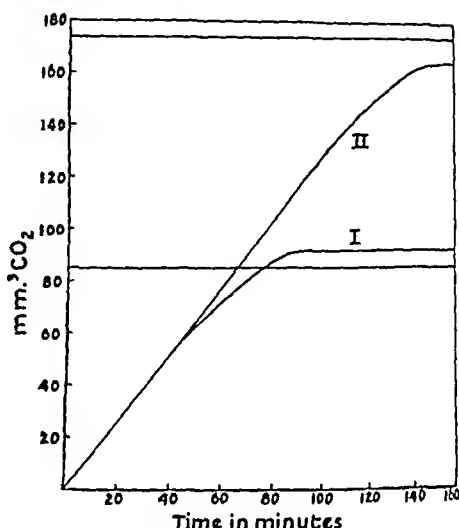
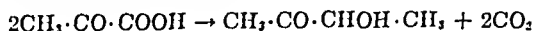


FIG. 2

FIG. 1. The CO_2 equivalence of α -ketoglutaric acid. The additions in each manometer vessel were 2 cc. of enzyme, dialyzed overnight against $\text{m}/7$ citrate buffer, pH 6.0, 0.2 cc. of 0.5 M phosphate buffer, pH 6.0, 0.1 cc. of 0.5 per cent MgSO_4 , and 0.1 cc. of 0.1 per cent diphosphothiamine. The final volume was made up to 3.0 cc. with water. Experiment I, substrate = 0.1 cc. of 0.057 M α -ketoglutarate; CO_2 evolved = 125 c.mm., theoretical CO_2 evolution = 128 c.mm. Experiment II, substrate = 0.2 cc. of 0.057 M α -ketoglutarate; CO_2 evolved = 250 c.mm., theoretical CO_2 evolution = 254 c.mm.

FIG. 2. The CO_2 equivalence of pyruvic acid. The additions in each manometer vessel were 2 cc. of enzyme, dialyzed overnight against $\text{m}/7$ citrate buffer, pH 6.0, 0.2 cc. of 0.5 M phosphate buffer, pH 6.0, 0.1 cc. of 0.5 per cent MgSO_4 , and 0.1 cc. of 0.1 per cent diphosphothiamine. The final volume was made up to 3.0 cc. with water. Experiment I, substrate = 0.1 cc. of 0.038 M pyruvate; CO_2 evolved = 92 c.mm., theoretical CO_2 evolution = 85 c.mm. Experiment II, substrate = 0.2 cc. of 0.038 M pyruvate; CO_2 evolved = 164 c.mm., theoretical CO_2 evolution = 174 c.mm.

Quantitative studies showed that pyruvate disappearance was equivalent to CO_2 production and that the moles of acetylmethylcarbinol formed were half as great as the moles of pyruvate which disappeared. The evidence for these statements is given in Table II. The equation for the reaction may, therefore, be written



Decarboxylation of α -Ketobutyric Acid—The product of decarboxylation of α -ketobutyric acid was identified as propionin by forming the 2,4-dinitrophenylosazone. By analogy with pyruvate, the reaction probably proceeds according to the equation



Action of the Enzyme on Acetaldehyde—Because α -ketoglutaric acid underwent simple decarboxylation, it seemed possible that acetaldehyde and propionaldehyde might be intermediaries in the formation of acetoin and propionin, respectively. The action of the enzyme on these aldehydes was therefore tested. Acetaldehyde was indeed found to undergo condensation to acetoin. No reaction was observed if the enzyme was boiled

TABLE II
Balance Sheet for Decarboxylation of Pyruvate

The theoretical values for the ratio of Columns 4:3 and of Columns 6:3 are 0.50 and 1.00 respectively. All concentrations are expressed in equivalents of c.mm. of CO_2 .

Initial amount of pyruvate (1)	Final amount of pyruvate (2)	Pyruvate disappearance (3)	Acetoin formation (4)	Ratio (4) (3) (5)	CO_2 production (6)	Ratio (6) (3) (7)
c.mm.	c.mm.	c.mm.	c.mm.		c.mm.	
16,960	3760	13,200	6180	0.47		
12,480	5630	6,850	3200	0.47		
16,960	5100	11,860	5980	0.50		
637	427	210			202	0.96
639	294	345			304	0.88
639	289	350			300	0.86
Average.	0.48		0.90

or if diphosphothiamine was not added to the system. The rate of formation of acetoin from acetaldehyde at pH 6 was, however, too slow to account for the complete disappearance of acetaldehyde if it were the first product formed in the decarboxylation of pyruvic acid. Furthermore, propionaldehyde was not attacked by the enzyme. Crotonaldehyde and acetone likewise were inactive. If, however, an aldehyde was added to the enzyme in the presence of an α -keto acid, a marked effect was observed and the aldehyde could readily be proved to enter into the reaction.

Action of the Enzyme on Pyruvate and Aldehyde—The data in Table III show that the rate of evolution of CO_2 from pyruvate and acetaldehyde together was about 4 times that obtained with pyruvic acid alone. The product of the reaction was acetoin. Quantitative determinations of CO_2

evolved, acetoin formed, and pyruvate and acetaldehyde removed during the course of a typical experiment are summarized in Table IV. The figures show that after correction for the acetaldehyde which disappears and the acetoin which is formed in the absence of added pyruvate, the

TABLE III

Influence of Acetaldhyde on Rate of CO₂ Evolution from Pyruvic Acid

The additions to the manometer vessels were 2.5 cc. of enzyme, 0.1 cc. of 0.6 M pyruvate, 0.1 cc. of M acetaldehyde, 0.1 cc. of 0.1 per cent diphosphothiamine, and 0.1 cc. of 0.5 per cent MgSO₄. The total volume of fluid was made up to 3.0 cc. with water. The gas was 95 per cent N₂, 5 per cent CO₂.

	CO ₂	
	10 min.	20 min.
	c.mm.	c mm.
Enzyme system + pyruvate + acetaldehyde....	145	298
Without acetaldehyde.	35	70
" magnesium... ..	96	191
" diphosphothiamine.....	18	36
" pyruvate.. ..	0	0
With boiled enzyme.	0	0

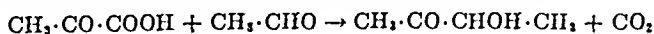
TABLE IV

Balance Sheet for Reaction of Pyruvate and Acetaldhyde

The changes in concentrations of reactants are expressed in all cases in equivalents of c.mm. of CO₂. The theoretical value is based on the CO₂ output.

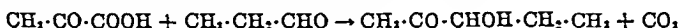
	Observed change in concentration	Per cent of theory
CO ₂ evolution...	+764	
Pyruvate disappearing... ..	-732	96
Acetaldehyde disappearing in presence of pyruvate.....	-1259	
" " " absence " " 	-590	
due to reaction with pyruvate	-669	88
Acetylmethylcarbinol formed in presence of pyruvate and acetaldehyde... ..	+802	
Acetylmethylcarbinol formed in presence of acetaldehyde only.....	+160	
Acetylmethylcarbinol formed due to reaction between pyruvate and acetaldehyde..	+642	84

acetoin formation, the acetaldehyde disappearance, and the pyruvate disappearance correspond approximately to the CO₂ evolution. The reaction is therefore described by the equation

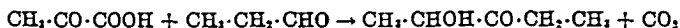


When propionaldehyde, butyraldehyde, or aldol was substituted for acetaldehyde, the rate of CO_2 evolution was 4.7, 3, and 4 times greater, respectively, than that found with pyruvate alone.

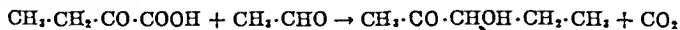
A quantitative study was made of the reaction between pyruvate and propionaldehyde. This reaction was more satisfactory from the analytical standpoint than that between pyruvate and acetaldehyde, since propionaldehyde alone was not attacked by the enzyme and no corrections were necessary for the action of the enzyme on propionaldehyde alone. In a typical experiment, 1090 c.mm. of CO_2 were produced, while pyruvate and propionaldehyde equivalent to 1100 and 1070 c.mm. of CO_2 respectively disappeared from the reaction mixture. Thus for 1.0 mole of CO_2 formed, 1.0 mole of pyruvate and 0.98 mole of propionaldehyde disappeared. The product of the reaction was isolated as the 2,4-dinitrophenylosazone and identified as either propionylmethylcarbinol or acetylmethylcarbinol. The reaction may therefore be written either



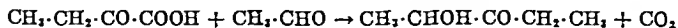
or



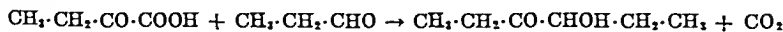
Action of the Enzyme on Aldehyde and α -Ketobutyric Acid—The addition of acetaldehyde or propionaldehyde to α -ketobutyric acid markedly increased the rate of CO_2 evolution as shown in Table V. The products were identified as the 2,4-dinitrophenylosazones. From acetaldehyde and α -ketobutyrate, either propionylmethylcarbinol or acetylmethylcarbinol was obtained. From propionaldehyde and α -ketobutyrate, propionin was obtained. Therefore, the reactions may be described by the following equations, either



or



and



Identification of Reaction Products

Succinic Semialdehyde—A large scale enzyme mixture was made up of 100 cc. of enzyme, 10 cc. of 0.5 M α -ketoglutarate, 5 cc. of 0.5 per cent MgSO_4 , and 5 cc. of 0.1 per cent diphosphothiamine. This mixture was incubated at 38° for 4 hours. At this time, the theoretical amount of CO_2 had been evolved as indicated by a manometric determination on an aliquot of the mixture incubated simultaneously. The mixture was deproteinized by

addition of an equal volume of 5 per cent trichloroacetic acid. The clear filtrate was made 2 *N* with respect to HCl and mixed with 200 cc. of 0.5 per cent 2,4-dinitrophenylhydrazine in 2 *N* HCl. After a few hours at room temperature, the precipitate was filtered off and washed with 2 *N* HCl and water. After two recrystallizations from hot water, the derivative melted at 196–197°, and showed no depression of the melting point when mixed with the 2,4-dinitrophenylhydrazone of succinic semialdehyde prepared synthetically (15). Both derivatives were identical in color and crystalline form.

Analysis— $C_{10}H_{10}O_8N_4$

Calculated. C 42.55, H 3.55, N 19.86

Found. " 42.78, 42.49, " 3.84, 4.10, " 20.13, 20.08

TABLE V

Decarboxylation of α -Ketobutyrate

The system was composed of 2.5 cc. of enzyme, 0.1 cc. of 0.5 per cent $MgSO_4$, and 0.1 cc. of 0.1 per cent diphosphothiamine. The amount added of both α -ketonic acid and aldehyde was 0.1 cc. of *M* solution. The experiments were carried out at pH 7.2 in phosphate-bicarbonate buffer and in 95 per cent N_2 , 5 per cent CO_2 .

	CO_2 in 60 min
	<i>c mm</i>
System + α -ketobutyrate	438
" + " + acetaldehyde.....	858
" + " + propionaldehyde.. . . .	1020
" (minus diphosphothiamine) + α -ketobutyrate + propionaldehyde.. . . .	204
System (enzyme was boiled) + α -ketobutyrate + propionaldehyde	0
System + pyruvate + acetaldehyde	528

Acetoin—The 2,4-dinitrophenylsazone of acetoin was isolated from the reaction mixture obtained by incubation of the enzyme system with (a) pyruvate as the only substrate, (b) pyruvate and acetaldehyde as the substrates, and (c) acetaldehyde as the only substrate. The results, except for yields, were identical; hence only one typical experiment is reported.

The reaction mixture consisted of 100 cc. of enzyme, 5 cc. of *M* pyruvate, 10 cc. of *M* acetaldehyde, 5 cc. of 0.5 per cent $MgSO_4$, and 5 cc. of 0.1 per cent diphosphothiamine. After 4 hours incubation at 38°, an equal volume of saturated ammonium sulfate was added and the mixture was heated to 90°. The precipitate was removed rapidly by suction filtration; excess acetaldehyde was thereby boiled off from the filtrate. The water-

clear solution was distilled practically to dryness, and the distillate was made 2 N with HCl and mixed with 1.5 volumes of 0.5 per cent 2,4-dinitrophenylhydrazine in 2 N HCl. The solution was heated on a steam bath for 2 hours, cooled, and filtered. The precipitate was washed exhaustively in turn with 2 N HCl, hot water, and hot 95 per cent alcohol. After several recrystallizations from hot pyridine or preferably nitrobenzene, the melting point (decomposition point) was constant at 315°. The osazone was identical with that of synthetic acetoin in color, crystalline structure, and melting point; there was no depression of the mixed melting point. Crystals of both the synthetic and natural osazones were brown when obtained from pyridine, and light orange from nitrobenzene. Both gave a purple color with alcoholic KOH and no color with aqueous NaOH.

Analysis—Sample dried at 100° and 1 mm. over P₂O₅:

C₁₁H₁₁N₅O₈. Calculated. C 43.04, H 3.16, N 25.11

Found. " 44.24, " 2.86, " 25.32

From the identification of the osazone, it is clear that the original product was either acetoin or diacetyl, since both would give the same derivative. On the basis of the following evidence, however, the product was identified as acetoin and not diacetyl: (a) The time necessary for the precipitation of the product with 2,4-dinitrophenylhydrazine was typical of osazone formation rather than hydrazone formation. Diacetyl precipitated with the reagent immediately at room temperature, whereas the decarboxylation product and acetoin required several days at room temperature or $\frac{1}{2}$ to 1 hour on the steam bath. (b) In the Voges-Proskauer reaction, the color was produced slowly as with acetoin rather than rapidly as with diacetyl. (c) Nickel dimethylglyoxime was not precipitated unless the decarboxylation product was first oxidized with ferric chloride. (d) The decarboxylation of pyruvic acid proceeded in the absence of oxygen; theoretically, diacetyl could not be formed without a simultaneous oxidation.

Propioin—The isolation of the 2,4-dinitrophenylosazone of propioin followed the previously described procedure for acetoin in which the distillate from the deproteinized reaction mixture was heated for 2 to 3 hours with an excess of 2,4-dinitrophenylhydrazine in 2 N HCl. The product was recrystallized from nitrobenzene. The derivative obtained from the decarboxylation of α -ketobutyric acid alone was identical with that obtained from the α -ketobutyric acid-propionaldehyde reaction; the yield in the latter case was much greater. Both derivatives were identical in every way with the osazone of synthetic propioin (16). The melting point (decomposition point) was 280° and there was no depression on mixing.

Analysis—C₁₁H₁₁N₅O₈. Calculated. C 45.56, H 3.82, N 23.62

Found. " 45.93, " 3.85, " 23.43

Mixed Acyloins—The product of the reaction of pyruvic acid and propionaldehyde was isolated as the 2,4-dinitrophenylosazone derivative prepared by the same procedure as that previously described for acetoin. After several recrystallizations from nitrobenzene, it had a constant melting point (decomposition point) at 270–271°. It was found to be identical with the 2,4-dinitrophenylosazone of synthetic propionylmethylcarbinol (17) in color (deep orange), crystalline structure, and melting point and there was no depression of the mixed melting point.

Analysis—Sample dried at 100° and 1 mm. over P₂O₅

C₁₇H₁₆N₈O₈. Calculated. C 44.34, H 3.51, N 24.34

Found. " 45.17, " 4.11, " 24.30

The identification of this osazone establishes the original product as either propionylmethylcarbinol or acetyl ethylcarbinol but does not distinguish between them.

The osazone obtained from the α -ketobutyric acid-acetaldehyde reaction was identical also with the osazone of synthetic propionylmethylcarbinol. The melting point (decomposition point) was 270–271° and there was no depression of the mixed melting point. This osazone apparently contained a small amount of the acetoin derivative.

Analysis—C₁₇H₁₆N₈O₈. Calculated. C 44.34, H 3.51, N 24.34

Found. " 44.15, " 3.37, " 24.73

Voges-Proskauer Reaction—The colors obtained in the Voges-Proskauer reaction provided a rapid means of identifying the products of the reaction, and these were confirmed with the synthetic acyloins. The solutions to be tested contained between 50 and 200 γ of substrate in 1.5 cc. and this was mixed with 2 cc. of 40 per cent sodium hydroxide and 0.1 cc. of 5 per cent creatine. The solution was shaken vigorously until maximum color was obtained. Acetoin gave a maximum cherry-red color in 5 minutes, propionylmethylcarbinol a purple color in 10 to 15 minutes, and propionin a pale olive-green color in 20 to 30 minutes.

Properties of the Enzyme

Source of Enzyme—Of all the tissues tested, pig heart muscle proved to be the most satisfactory source of the enzyme. Active preparations could be obtained, however, from pigeon breast muscle, rabbit skeletal muscle, rabbit liver, pig liver, and pig kidney, as determined by CO₂ evolution from pyruvic acid and the appearance of a positive Voges-Proskauer reaction.

Stability of Enzyme—The yield of enzyme from pig heart varied and seemed to be lower if the hearts were frozen. The activity of the enzyme preparation was maintained unimpaired for about a week at 0°. Substi-

tution of phosphate or citrate buffer for β -glycerophosphate in the original extraction resulted in preparations of little or no activity. At the acid precipitation stage considerable loss of activity occurred if the temperature was not kept low.

Exposure of the enzyme to temperatures above 50° led to rapid inactivation. For example, 1 minute at 50° led to 15 per cent inactivation, 1 minute at 70° to 100 per cent inactivation of the pyruvate system. 0.01 M malonate, 0.002 M iodoacetate, 0.0001 M cupric sulfate, 0.002 M arsenite, 0.01 M sodium fluoride, 0.05 per cent sulfanilamide, and a saturated solution of capryl alcohol had no appreciable inhibitory action on the decarboxylation of pyruvate.

The enzyme preparation had no action on oxalacetic acid, mesoxalic acid, α -ketocaproic acid, or phenylpyruvic acid; the latter two were tested with added aldehydes without effect.

Though most of the experiments were carried out at pH 6.0, the decarboxylation of both pyruvic acid and α -ketoglutaric acid took place over a pH range of 6 to 8. The formation of acetoin from acetaldehyde alone was 2 to 3 times greater at pH 7.4 than at pH 6. It did not take place above pH 8.

In the presence of pyruvate or α -ketoglutarate, the rate of CO₂ evolution decreased very little with time but continued almost linearly for 4 or more hours. This is in striking contrast to the behavior of yeast carboxylase. The insensitivity of the animal enzyme to copper also differentiates it from the yeast enzyme.

Rôle of Diphosphothiamine—As already shown in Table I, the enzyme preparation found to be most satisfactory required the addition of diphosphothiamine and of magnesium or manganese for maximum activity. If the acid precipitation was carried out at pH 4.8 to 5.0 instead of at 4.6, the preparation (which did not separate clearly on centrifugation) did not show an increased rate of CO₂ production from pyruvate on addition of diphosphothiamine. Furthermore, exhaustive washing of the original muscle mince did not reduce the bound diphosphothiamine. It seems, therefore, that the coenzyme is bound to the protein and is not dissociated to a significant extent at physiological pH values, but that some dissociation occurs at pH 4.6. The precipitate obtained at this pH, however, shows some activity without added diphosphothiamine, and also contains some bound coenzyme.

To obtain a preparation which showed no activity in the decarboxylation of pyruvate unless supplemented with diphosphothiamine, the enzyme preparation at the acetic acid precipitation stage (pH 4.6) was allowed to stand at 0° for 6 hours. 3 volumes of cold water were added. The precipitate was then centrifuged off and resuspended as usual in the phosphate-

bicarbonate mixture. Fig. 3 shows the relation between the rate of CO_2 evolution from pyruvic acid and the concentration of diphosphothiamine in the reconstructed system. The maximum rate was reached with a final concentration of 2 γ of diphosphothiamine per cc. Thiamine could not be substituted for diphosphothiamine.

The procedure adopted for splitting the pyruvic enzymes led to considerable loss of activity, and the preparations were completely inactivated toward α -ketoglutaric acid. To retain catalytic activity with α -ketoglutaric acid, contact with the acetic acid solution had to be reduced to a maximum of 10 minutes at pH 4.8. After two precipitations under these

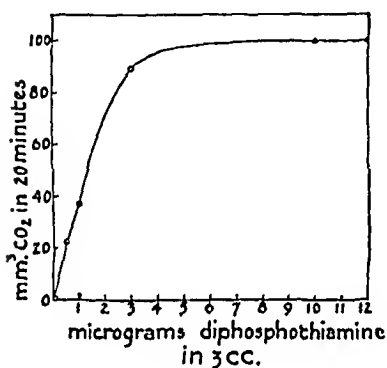


FIG. 3

FIG. 3. Dependence of CO_2 evolution on concentration of diphosphothiamine in reconstructed pyruvate system. Each manometer vessel contained 2 cc. of heart enzyme, 0.4 cc. of 0.1 M pyruvate, 0.2 cc. of M acetaldehyde, and 0.1 cc. of 0.5 per cent MgSO_4 . Water was added to a final volume of 3.0 cc.

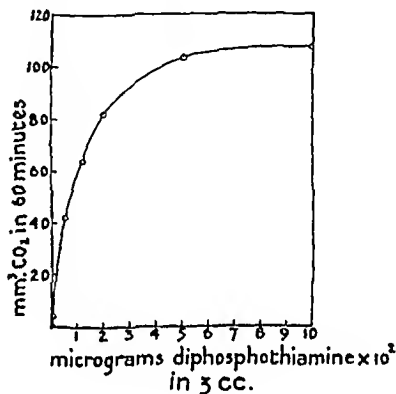


FIG. 4

FIG. 4. Dependence of CO_2 evolution on concentration of diphosphothiamine in reconstructed α -ketoglutarate system. Each manometer vessel contained 2.5 cc. of heart enzyme, 0.2 cc. of 0.5 M α -ketoglutarate, and 0.1 cc. of 0.5 per cent MgSO_4 . Water was added to a final volume of 3.0 cc.

conditions, the preparation showed no activity with α -ketoglutaric acid in the absence of added diphosphothiamine. Fig. 4 shows the relation between the rate of CO_2 evolution from α -ketoglutaric acid and the concentration of diphosphothiamine in the reconstructed system. Maximum reaction velocity was attained when the concentration of diphosphothiamine was 300 γ per cc. as compared to the 2 γ per cc. required by the pyruvic enzyme. Thus the ease of combination of the prosthetic group with the protein is very different for the two systems. This observation suggests that the two enzymes are not identical.

That the condensation of acetaldehyde to acetoin is due to a third dis-

tinct though similar enzyme is indicated by the fact that preparations from heart muscle have been obtained which showed no diphosphothiamine effect on the decarboxylation of pyruvate or α -ketoglutarate, but which were inactive in condensing acetaldehyde unless supplemented with diphosphothiamine. The dependence of the acetaldehyde-condensing enzyme, which may be referred to as aldehyde ketolase, on diphosphothiamine is shown in Table VI. For maximum activity, about 30 γ of diphosphothiamine per cc. were required.

Rôle of Certain Inorganic Ions—Besides diphosphothiamine, the pyruvic enzyme also required a divalent cation. The complete removal of the metal component from the enzyme was technically more difficult than the removal of the diphosphothiamine. The acetic acid precipitation and resuspension in the buffer mixture had to be repeated at least three times

TABLE VI
Components of Acetaldehyde-Condensing Enzyme

The additions were 10 cc. of enzyme and 0.8 cc. of M acetaldehyde. The final volume was made up to 15 cc. The experiment was carried out for 3 hours at 38°. Acetoin was estimated as nickel dimethylglyoxime.

Enzyme	Boiled enzyme	Diphospho- thiamine	Thiamine	Acetaldehyde	Nickel dimethylglyox- ime
		γ	γ		mg.
+	0	4000	0	+	7.5
+	0	4000	0	0	0
0	+	4000	0	+	0
+	0	0	0	+	0
+	0	400	0	+	7.2
+	0	40	0	+	1.5
+	0	0	4000	+	0

before the rate of CO_2 evolution from pyruvate in the absence of added divalent cations approached zero. As a result of this drastic treatment, much of the original enzyme activity was destroyed. In a typical experiment, the split enzyme plus pyruvate, acetaldehyde, and diphosphothiamine gave 10 c.mm. of CO_2 in 1 hour. When 30 γ of magnesium per cc. were added, 52 c.mm. of CO_2 were obtained; and when 30 γ of manganese per cc. were added, 58 c.mm. of CO_2 were obtained in 1 hour. A concentration of 3 γ per cc. of magnesium or manganese gave rates about 80 per cent as high as those obtained with 30 γ per cc.

Both inorganic phosphate and arsenate showed a stimulating effect on the activity of the decarboxylating enzymes. In the experiment recorded in Table VII, the enzyme was prepared in the usual manner except that the acid precipitate was suspended in citrate buffer at pH 6.0, and dialyzed

against 0.25 M citrate buffer. Inorganic phosphate was completely removed, but the enzyme retained its activity. The addition of phosphate had an accelerating action, though its presence was not essential for activity.

TABLE VII

Stimulating Effect of Inorganic Phosphate on Decarboxylation of Pyruvic and α -Ketoglutaric Acids

The following were the additions in each manometric run: 2 cc. of enzyme dialyzed exhaustively against 0.25 M citrate buffer, pH 6.0, 0.1 cc. of 0.5 per cent MgSO_4 , 0.1 cc. of 0.1 per cent diphosphothiamine, and 0.1 cc. of M α -ketonic acid. The phosphate and arsenate buffers were adjusted to pH 6.0.

Substrate	Additions	CO ₂ in 60 min.
		<i>c.mm.</i>
Pyruvate	None	164
"	0.2 cc. 0.5 M phosphate	264
"	0.2 " 0.05 " "	180
"	0.2 " 0.5 " arsenate	255
α -Ketoglutarate	None	186
"	0.2 cc. 0.5 M phosphate	278

TABLE VIII

Acetoin-Forming Enzyme of Yeast

The additions were 15 cc. of enzyme (split or unsplit), 2.4 cc. of 0.1 M pyruvate, 1.2 cc. of M acetaldehyde, 1 cc. of 0.1 per cent diphosphothiamine, and 1 cc. of 0.5 per cent MgSO_4 . The final volume was made up to 23 cc. with water.

Split enzyme	Mg	Diphospho- thiamine	Thiamine	Unsplit enzyme	Pyruvate	Acetal- dehyde	Dimethyl- glyoxime
							<i>mg.</i>
—	—	—	—	+	+	+	7.8
—	—	—	—	+	+	0	0.4
—	—	—	—	+	0	+	0.0
+	+	+	0	—	+	+	3.5
+	+	0	0	—	+	+	0
+	0	+	0	—	+	+	0
+	+	0	+	—	+	+	0

Acetoin Formation by Yeast

The mechanism of acetoin formation by yeast has been a controversial subject. A crude enzyme preparation with properties very similar to the animal enzyme was obtained from yeast by the following method. 100 gm. of air-dried brewers' yeast were rubbed up with 300 cc. of M/15 phosphate buffer, pH 7.2, and incubated for 1 hour at 37°. The mixture was diluted with 400 cc. of water and centrifuged. 38 gm. of ammonium

sulfate were added for each 100 cc. of supernatant fluid. The precipitate was dissolved in 300 cc. of $M/15$ phosphate buffer, pH 7.2, and the ammonium sulfate precipitation was repeated. The precipitate was finally dissolved in 40 cc. of 0.1 M phosphate buffer, pH 7.2.

Table VIII shows that this enzyme preparation formed acetoin from pyruvate and acetaldehyde, but not from acetaldehyde alone, and very little from pyruvate alone. The yeast enzyme preparation contained considerable amounts of carboxylase, but at pH 7.2 and under the conditions of the experiment, the activity of the carboxylase was found to be only a very small fraction of its activity at pH 6.0 under optimal conditions.

The yeast enzyme was resolved into its component parts by the ammoniacal ammonium sulfate procedure described for carboxylase by Green *et al.* (4). Table VIII shows that the split enzyme produced acetoin from pyruvate and acetaldehyde only in the presence of both magnesium ions and diphosphothiamine. Thiamine was inactive in the reconstructed system.

DISCUSSION

Acetoin production has long been known to occur in yeasts, molds, bacteria, plants, and animal tissues (see Neuberg and Simon (18) for an extensive review of the earlier literature). Gorr (19) isolated acetoin as the *p*-nitrophenylosazone derivative from minced heart muscle which had been incubated with pyruvic acid. Recently Tanko *et al.* (20) showed that other animal tissues such as skeletal muscle, liver, and kidney produced acetoin from pyruvic acid. They further established that acetaldehyde increased the rate of acetoin formation from pyruvate and that the acetoin formed was optically active.

Together with diacetyl and 2,3-butyleneglycol, acetoin has been identified as a minor constituent of normal human urine (21, 22) and of the blood of higher animals, ox, sheep, horse, pig (23, 24).

Acetoin formation by the animal and yeast enzymes seems to be different from that of the cell-free extract of *Aerobacter aerogenes* described by Silverman and Werkman (25). This bacterial enzyme which forms acetyl-methylcarbinol from pyruvate is reported to be inactive above pH 7. The animal enzyme is active up to pH 8. Furthermore, acetaldehyde does not enter into the reaction in the bacterial system.

SUMMARY

A suspension of protein obtained from washed heart muscle has been found to catalyze the decarboxylation of α -ketoglutaric acid to succinic semialdehyde, the decarboxylation of pyruvic acid with the formation of acetoin, and the decarboxylation of α -ketobutyric acid with formation of propion. These reactions are catalyzed by diphosphothiamine-metallo-

proteins which under controlled acid conditions can be reversibly resolved into their component parts.

Acetaldehyde added to the pyruvate system is utilized in the formation of acetoin, and other aldehydes give analogous ketols. Similarly, α -keto-butyric acid and aldehydes give analogous acyloins.

Some of the properties of the enzyme system involved have been described.

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BIBLIOGRAPHY

1. Peters, R. A., *Biochem. J.*, **30**, 2206 (1936). Peters, R. A., and Thompson, R. H. S., *Biochem. J.*, **28**, 916 (1934). Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, **33**, 1109, 1980 (1939).
2. Pyke, M., *Biochem. J.*, **34**, 1341 (1940).
3. Green, D. E., Westerfeld, W. W., Vennesland, B., and Knox, W. E., *J. Biol. Chem.*, **140**, 683 (1941).
4. Green, D. E., Herbert, D., and Subrahmanyam, V., *J. Biol. Chem.*, **135**, 795 (1940).
5. Dewan, J. G., and Green, D. E., *Biochem. J.*, **32**, 626 (1938). Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **125**, 171 (1938).
6. Clift, F. P., and Cook, R. P., *Biochem. J.*, **26**, 1788 (1932).
7. Stahley, G. L., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **10**, 205 (1936).
8. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 654 (1937).
9. Neuberg, C., and Ringer, M., *Biochem. Z.*, **71**, 228 (1915).
10. Wohl, A., and Oesterlin, C., *Ber. chem. Ges.*, **34**, 1139 (1901).
11. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
12. Shemin, D., and Herbst, R. M., *J. Am. Chem. Soc.*, **60**, 1954 (1938).
13. Wendel, W. B., *J. Biol. Chem.*, **94**, 717 (1931-32).
14. O'Meara, R. A. Q., *J. Path. and Bact.*, **34**, 401 (1931).
15. Carriere, E., *Ann. chim.*, **17**, 38, 70 (1921).
16. Bouveault, L., and Loequin, R., *Bull. Soc. chim.*, **35**, 629 (1906).
17. Gauthier, D. D., *Compt. rend. Acad.*, **152**, 1100 (1911).
18. Neuberg, C., and Simon, E., in Nord, F. F., and Weidenhagen, R., *Ergebnisse der Enzymforschung*, Leipzig, **2**, 118 (1933).
19. Gorr, G., *Biochem. Z.*, **254**, 12 (1932).
20. Tanko, B., Munk, L., and Abonyi, I., *Z. physiol. Chem.*, **264**, 91 (1940).
21. Schmalzfuss, H., Schaaake, H., and Barthmeyer, H., *Z. physiol. Chem.*, **200**, 169 (1931).
22. Schmalzfuss, H., *Z. physiol. Chem.*, **188**, 279 (1930).
23. Lemoigne, M., and Monguillon, P., *Compt. rend. Acad.*, **191**, 80 (1930).
24. Schmalzfuss, H., Hinseh, W., and Schmalzfuss, H., *Z. physiol. Chem.*, **227**, 247 (1934).
25. Silverman, M., and Werkman, C. H., *J. Biol. Chem.*, **138**, 35 (1941).

TITRIMETRIC MICRODETERMINATION OF CHLORIDE, SODIUM, AND POTASSIUM IN A SINGLE TISSUE OR BLOOD SAMPLE*

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During a study of electrolyte distribution in muscles, it became necessary to determine water, dry weight, sodium, potassium, and chloride in the same small tissue aliquot, weighing 50 to 200 mg. or less wet weight. Previously described methods of chloride and base determinations on the same sample were found unsuitable for our purposes. The electro dialysis methods of Joseph and Stadie (1) and Oster *et al.* (2, 3) do not permit analyses on small aliquots of tissue; while the method of Dean and Fishman (4), although satisfactory for chloride determination by the Volhard procedure, gives a poorer end-point than the mercurimetric method. Moreover, Dean and Fishman's paper does not elucidate the method of analysis of sodium and potassium.

The method of choice was such that it could be easily employed in an ordinary biological laboratory, requiring easily obtainable and inexpensive equipment.

In our method, chloride is driven off as hydrogen chloride from the weighed dried sample, which is digested in concentrated sulfuric acid by a micro diffusion technique modified from the method of Dean and Fishman (4), and collected in potassium hydroxide. The resulting KCl is determined by a mercurimetric titration with diphenylcarbazone as an indicator, modified from the method described by Dubsky and Trtilek (5), whose method was recently adapted by Schales and Schales (6) in the determination of serum chloride.

Potassium and sodium are determined in the digest after evaporation and ashing. Potassium is precipitated as the chloroplatinate, and reduced with sodium formate, according to the method of Cunningham, Kirk, and Brooks (7). The liberated chloride is then determined mercurimetrically.

Sodium is determined by a modification of the method of Dregus (8), being precipitated as sodium zinc uranyl acetate according to Barber and

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Kolthoff (9), and the dissolved precipitate titrated with sodium hydroxide, phenolphthalein being used as indicator.

The method was standardized on pooled samples of blood serum ranging from 0.5 to 1.0 gm., and on samples of vitamin-free casein (S. M. A. Corporation). Some determinations on rat serum and diaphragm muscle are included. The serum and casein were chosen rather than muscle tissue because of their constant electrolyte values. Known amounts of sodium, potassium, and chloride were added to other samples, and the per cent recoveries determined.

Reagents—

1. Strong KOH, 50 per cent; store in a paraffined bottle.
2. Concentrated H_2SO_4 , c.p.
3. Dilute HNO_3 (0.2 N and 0.01 N).
4. Phenolphthalein, 1 per cent in methanol; 0.05 per cent in methanol.
5. H_2O_2 , 30 per cent (superoxol).
6. Diphenylcarbazone indicator, 1 per cent in methanol (c.p.), Eastman No. 4459; store in a dark bottle and keep in the refrigerator. It is stable at least 6 months.
7. Mercuric nitrate standard. Dissolve 1.8 gm. of the hygroscopic analytical grade salt in water, add 10 ml. of 1 N HNO_3 , and make up to 1 liter. Standardize against KCl or NaCl.
8. Sodium, potassium, and chloride standards. Dissolve 0.0100 M of dried analytical grade KCl and NaCl and make up to 1 liter.
9. Sodium hydroxide, CO_2 -free standard solution. See Kolthoff and Sandell (10). Standardize against Bureau of Standards biphthalate. The strength of the NaOH may be 0.01 to 0.10 N, according to the amount of sodium to be titrated (see the "Procedure").
10. Sucrose, analytical grade crystals.
11. Zinc uranyl acetate, sodium-precipitating reagent (see Kolthoff and Sandell (10)). (A) Uranyl acetate $\cdot 2\text{H}_2\text{O}$, 20 gm.; acetic acid, 30 per cent, 6 gm.; water, 49 gm. (B) Zinc acetate $\cdot 3\text{H}_2\text{O}$, 30 gm.; acetic acid, 30 per cent, 3 gm.; water, 32 gm. Mix A and B, add 1 drop of 0.01 N NaCl, and leave in the dark. Centrifuge or filter before use if a film forms on the surface.
12. Sodium wash fluids. (A) Acetic acid-ethanol mixture. Mix 10 ml. of glacial acetic acid and 90 ml. of 95 per cent ethanol, and shake with a small amount of sodium zinc uranyl acetate. Filter before use. It is stable for 1 week in the dark. (B) Sodium zinc uranyl acetate. Dissolve 20 mg. of NaCl in 1 ml. of H_2O , and add 10 ml. of the zinc uranyl acetate reagent (Reagent 11). Filter through a sintered glass filter after stirring and allowing to stand 1 hour. Wash several times with glacial acetic acid, followed by ethanol, then ether. Keep dry and in the dark. (C)

Ethanol, 95 per cent, saturated with sodium zinc uranyl acetate (Reagent 12B). (D) Diethyl ether, c.p., water-free.

13. Water, CO_2 -free. Boil distilled water. Store in a Pyrex bottle with a 2-hole rubber stopper connected with a soda lime drying tube and a delivery siphon.

14. Potassium chloroplatinate solution. Dissolve 50 mg. of the dried salt in 100 ml. of H_2O . To prepare the chloroplatinate, add an equivalent amount of a concentrated solution of KCl to chloroplatinic acid, add ethanol, filter through sintered glass, wash several times with 95 per cent ethanol, then ether, and dry.

15. Potassium wash fluids. (A) 80 per cent ethanol wash fluid. 5 volumes of 95 per cent ethanol, and 1 volume of H_2O saturated with potassium chloroplatinate (Reagent 14). (B) 95 per cent ethanol saturated with potassium chloroplatinate (Reagent 14).

16. Potassium-precipitating reagent. 20 per cent chloroplatinic acid, analytical grade. 1 gm. of chloroplatinic acid made up to 5 ml. with 1 N HCl. (Save waste platinates for recovery of platinum.)

17. Chloroplatinate-reducing reagent. Sodium formate, 0.2 N , analytical grade.

Apparatus

Filter Sticks—For filtration and washing of the sodium and potassium precipitates, we use asbestos filter sticks described by Emich ((11) p. 71). Where it was necessary to save the filtrate, an apparatus similar in principle to the one described by Emich ((11) p. 76) was used (see Fig. 1). In Fig. 1, *A* is 1 mm. glass tubing, blown out at the end and filled with a small amount of asbestos at *B*. The amount is easily established by experience and depends on the type of precipitate to be filtered. The asbestos plug is washed by drawing distilled water through it by aspiration, and dried. *C* is a small glass vial of suitable dimensions, in our case 65×20 mm. *D* is a thick walled glass cylinder or test-tube, 100×30 mm.

Burettes—Ordinary calibrated micro burettes are used for titrations when the volume of the solution was larger than 0.5 ml. For smaller volumes we use a hypodermic syringe attached to a vernier micrometer caliper screw mechanism. A similar apparatus has been described by Trevan (12), Kryukov and Kalarova (13), Scholander (14), Dean,¹ and others. Fig. 2 illustrates our particular design of such an arrangement. Scholander used a mercury-driven burette, while ours is air-driven. In Fig. 2, *E* is a Becton-Dickinson 1 ml. tuberculin syringe, the plunger of which is driven by the mechanism of the vernier micrometer caliper, re-

¹ Dean, R. B., unpublished.

moved from its curved arm and set in a brass holder, *G*, by means of a tight metal bushing. The syringe is clamped to the same holder by means of a removable brass plate, held in place with brass bolts. The end of the micrometer plunger is pointed in a lathe and pushes against a small metal disk cemented to the end of the plunger of the syringe and held centered there by operating against a small depression made in the disk. Ordinary capillary glass tubing is attached to the syringe by a small piece of rubber suction tubing and held tightly by wiring. If the syringe is Pyrex, it is sometimes more convenient to fuse this tubing on, but this invites

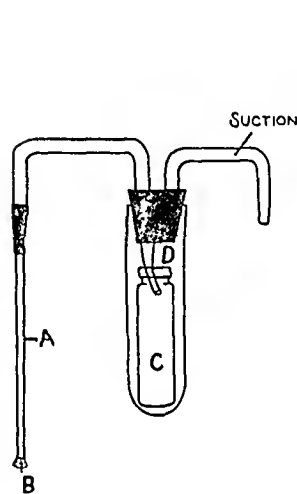


FIG. 1

FIG. 1. Filter stick filtration assembly. *A*, 1 mm. glass tubing; *B*, asbestos plug; *C*, glass vial, 65 × 20 mm.; *D*, glass tube, 100 × 30 mm.

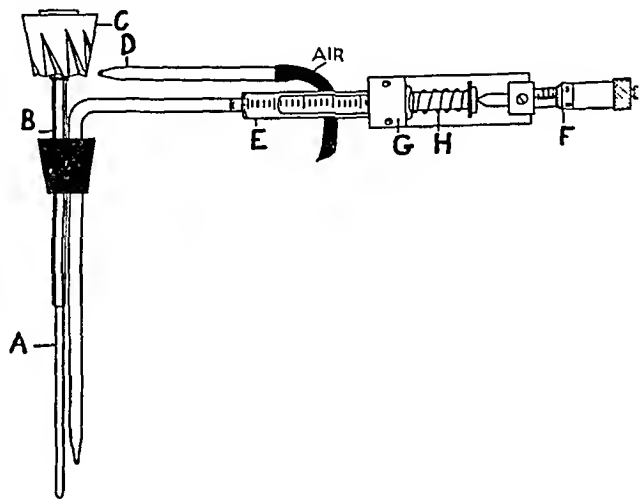


FIG. 2

FIG. 2. Micro burette assembly. *A*, glass stirrer; *B*, glass tube bushing; *C*, cork air-turbine; *D*, air jet; *E*, Beeton-Dickinson 1 ml. tuberculin syringe; *F*, vernier micrometer caliper; *G*, brass holder; *H*, steel spring.

breakage. Fluid enters the capillary tubing more easily if the attached end of the capillary is flanged out somewhat. The steel spring, *H*, forces the plunger of the syringe back when the burette is filled by screwing out the micrometer screw. The solution to be titrated is stirred by the glass rod, *A*, attached to a cork air-turbine, *C*, which rotates in the glass bushing, *B*, lubrication between the cork and bushing being provided by a small steel washer which rests on top of the bushing. The rate of stirring is easily adjustable by controlling the force of the air jet. An adjustable illumination white light for titration is conveniently made by mounting a 6 to 12 volt automobile headlight lamp set in front of a metal reflector in

a small microscope lamp housing, a daylight filter being used in combination with a white glass filter made from ordinary white enameled glass. This lamp can be energized from a transformer and has quite a long life.

Calculations for the calibration of the syringe burette are as follows:

Let X_1, X_2, X_3, \dots , etc., be the ml. of standard solution, *e.g.* chloride, delivered from a Krogh syringe pipette (see below), or weighed to 0.1 mg.

Let Y_1, Y_2, Y_3 , etc., be the readings in inches on the micrometer vernier scale after the chloride has been titrated with, for example, standard mercuric nitrate.

The slope of the titer, *i.e.* ml. per inch, equals

$$\frac{X_1 - X_2}{Y_1 - Y_2} = \frac{X_1 - X_2}{Y_1 - Y_2} = \frac{X_2 - X_3}{Y_2 - Y_3}$$

if the burette has a uniform bore and the vernier scale is accurately calibrated.

From the equation of a straight line, $Y = aX + b$.

Hence b is a constant indicating any correction for the errors of the burette or the indicator end-point, and $a = (Y_1 - Y_2)/(X_1 - X_2)$, a second constant depending upon the concentrations of the solutions.

Sample Calculations— $X_1 = 0.400$ ml. (delivered from a Krogh syringe pipette); $X_2 = 0.198$ ml. (delivered from a Krogh syringe pipette); $Y_1 = 0.456$ inch on the vernier scale; $Y_2 = 0.225$ inch on the vernier scale. Hence the slope equals

$$\frac{X_1 - X_2}{Y_1 - Y_2} = \frac{0.400 - 0.198}{0.456 - 0.225} = \frac{0.202}{0.231} = 0.875 \text{ ml. per inch}$$

The normality of the standards being known, the ml. per inch can be easily converted to microequivalents per inch.

$$a = \frac{0.231}{0.202} = 1.14, \quad \text{and} \quad b = Y - aX = 0.456 - (1.14 \times 0.400) = 0$$

Therefore the error is 0.

This calibration is rapid and simple, and can be run for each new standard solution by choosing two Krogh syringe pipette deliveries of different volumes. We usually ran such a calibration each time we ran a series of new determinations, as a routine procedure.

Pipettes—Ordinary calibrated, quantitative delivery pipettes were used for volumes larger than 1 ml. For smaller volumes, we found it very convenient to use Krogh syringe pipettes as described by Krogh and Keys (15) which were made from 1 ml. Becton-Dickinson Pyrex tuberculin syringes with a fused capillary tip, as shown in Fig. 3. We preferred the capillary glass tip rather than the metal hypodermic needle tip described

by Krogh and Keys. In Fig. 3, *A* is the tuberculin syringe, held in a leucite clamp near its base. *D* is a grooved piece of metal cemented to the plunger of the syringe, the grooves fitting against the two brass rods one of which is labeled *B*. This acts as a guide when the plunger is inserted or pulled out. *C* is a long set screw which can be set for delivery of any desired volume. Our deliveries were rapid and accurate to 0.03 mg.

Micro Diffusion Apparatus—See the procedure for chloride determination. Ordinary ground glass-stoppered (matched) weighing bottles are

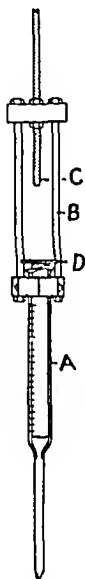


FIG. 3

FIG. 3. Krogh syringe pipette. *A*, Beeton-Dickinson 1 ml. Pyrex tuberculin syringe, with fused tip; *B*, brass guide rods; *C*, set screw for calibrating; *D*, metal disk, grooved at the edges to fit the guide rods.

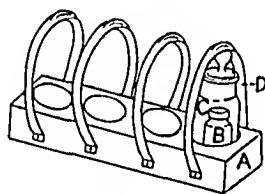


FIG. 4

FIG. 4. Clamps and holders for micro diffusion procedure. *A*, wooden block holder; *B*, small vial, or diffusion well; *C*, glass weighing bottle, 40 × 25 mm.; *D*, phosphor bronze strap springs.

used. The dimensions are 40 × 25 mm. Pyrex weighing bottles with "universal" joints are unsuitable, as the fits are not satisfactory. A small vial, with a flanged lip, is inserted into the weighing bottle, resting on the bottom, and of such proportions that its lip does not touch the stopper or sides of the weighing bottle. The bottles are incubated at 60–70°, and to prevent loss of gases by expansion, the stoppers are clamped tightly by phosphor bronze springs pivoted to a wooden block as shown in Fig. 4. By pressing the sides of these springs, pressure is released and

the bottles may be removed from the wooden blocks by pushing the spring through a small arc on its pivot screws. The stoppers should be removed before the bottles cool, to prevent freezing the ground glass joint as the inside gas pressure decreases. In Fig. 4, *D* is a phosphor bronze strap spring, with a groove at the top to fit the stopper of the weighing bottle. *A* is the wooden block, *C* is the bottle, and *B* is the small vial or center diffusion well.

Procedure

Chloride Determination—A sample of tissue is taken from the diaphragm of a rat decapitated with tin shears and bled. The rat is then eviscerated and the excess blood sponged away, the thorax opened, and the phrenic nerves cut, the connective tissue and membranes being trimmed away from the diaphragm, leaving it *in situ*. Clean scissors are used to cut out the diaphragm *in toto* along its edges of insertion along the body wall. The diaphragm is then placed on a fine grained filter paper, gently straightened out with forceps, and blotted manually with another filter paper. The diaphragm is left on the filter paper to facilitate trimming away the edges and the center membranes, and for division into two halves. The halves are removed from the paper, care being taken not to include adhering particles of the filter paper. The samples are rapidly weighed on the hook of a 500 mg. torsion balance and dropped into the weighed micro diffusion weighing bottles, which are then placed in the oven and dried at 105–110° for 6 to 12 hours or until they are of a relatively constant weight. The bottles are then cooled, dried, and weighed for water content. We have found that this procedure of removal and drying the muscle samples gives a quite consistent water content.

Known standard solutions, serum, or plasma samples are delivered into the micro diffusion bottles directly from a Krogh pipette, weighed, and dried to constant weight in the oven at 105–110°.

In the weighing bottle is placed the small removable center well (Fig. 4, *B*), containing 1 to 2 drops of 50 per cent KOH. The amount of KOH is determined by the total amount of diffusing acid gases, such as HCl, CO₂, SO₂, and so forth. Usually 1 to 2 drops of the 50 per cent solution constitutes a reasonable excess for the tissue aliquots used. The removable diffusion well must not be touched with the hands and must be perfectly clean. The flanged lip of the well must not touch the stopper of the weighing bottle or the sides. The stopper is then lubricated with concentrated H₂SO₄. 0.5 to 1.0 ml. of concentrated H₂SO₄ is added to the weighing bottle and it is quickly stoppered. The concentrated acid could be tipped in from a side chamber after stoppering, but we found the side chamber was an unnecessary added expense, and difficult to blow, because

Pyrex weighing bottles with good ground glass fits are not available. The bottle is then placed in the wooden block and the phosphor bronze spring clamps adjusted to clamp the stopper tightly, as depicted in Fig. 4. This is incubated in an oven at 65–70° for 6 hours, whereupon all the tissue chloride diffuses into the center well as HCl. Most of the HCl diffuses over within an hour, and all of it within 4 to 6 hours.

The center well is then removed from the weighing bottle with clean forceps and its outside surfaces washed with distilled water back into the H₂SO₄ digest in the weighing bottle, which is saved for the sodium and potassium analyses.

The excess KOH in the well is neutralized with approximately 0.2 N HNO₃, phenolphthalein (0.05 per cent in methanol) being used as indicator. Add a slight excess of HNO₃ and water to make the volume 1 ± 0.1 ml., and approximately 0.01 N in nitric acid.

Add 1 drop of 30 per cent H₂O₂ and 1 drop of the diphenylcarbazone indicator. Methanol is preferred to ethanol as a solvent for this dye, since it makes a more stable solution. The solution in the small well is titrated with mercuric nitrate (Reagent 7) until a permanent light blue color develops from the colorless solution. This is a very sensitive and sharp end-point.

An example of our results of the determination of chloride in vitamin-free casein is shown in Table I.

Table II illustrates results obtained on chloride determinations of rat blood serum. The blood samples were obtained through a large needle from the abdominal aorta in a clean dry 10 ml. syringe, the heart of the anesthetized animals being allowed to pump the blood into the syringe. The serum was obtained by centrifugation of the whole blood, and was not hemolyzed. The serum samples were pooled and kept under oil in the refrigerator until used. In the chloride determinations of rat plasma, it was not necessary to use syringe burettes, and ordinary 5 ml. burettes graduated in 0.01 ml. were used. The samples of serum ranged between 600 and 900 mg. Individual results between 100.3 and 102.1 microequivalents per gm. of serum averaged 101.2 ± 0.1 in twenty determinations. The serum samples thus contained 109.1 microequivalents per gm., while 7.83 microequivalents, or only 7 per cent of the total amount of chloride, were added, indicating the precision of the method.

Table III is a typical experiment of the determination of chloride in samples of human blood serum taken from a pooled supply of stored sterile serum.

The average in microequivalents per gm. of serum (milliequivalents per liter) is 101.50 ± 0.1 , the results ranging between 100.90 and 101.78.

Table IV illustrates the determination of muscle chlorides of the rat diaphragm, each figure representing a different rat.

In twenty-four samples of rat diaphragm muscle, we found the following ranges: per cent dry weight, 21.6 to 24.9; gm. of H_2O per kilo of wet weight, 751 to 777 (dried at 105–106° for 12 hours).

On adding chloride to the rat tissues (ten samples), we recovered, on the average, 100.8 per cent. Since there is considerable variation of muscle chloride from rat to rat, this recovery is as good as may be expected.

Sodium Determination—The sulfuric acid residue in the micro diffusion weighing bottles is quantitatively transferred to a platinum crucible and

TABLE I
Determination of Chloride in Vitamin-Free Casein

Casein analyzed	Chloride found		Cl added	Cl recovered	Recovery
	Micrometer readings				
mg.	inches	microequivalents	microequivalents	microequivalents	per cent
200	0 191	1.68			
200	0 188	1 66			
200	0.192	1 69			
200	0 188	1 66			
Average Cl per gm.		8 36 (to be subtracted from subsequent determinations of recoveries of added Cl)			
100	0 322	2 83	1 98	1 977	100.15
100	0 320	2 82	1 98	1 980	100.00
100	0 322	2 83	1 98	1 977	100.15
100	0 319	2 81	1 98	1 971	99 55
Comparison with Volhard titration, by same procedure. The titration was performed in an ice bath, ether being added near the end-point to clarify the solution of ppt, since stirring drives the ppt. into the ether layer					
46.4		2 35	1 95	1 96	100.5
47.2		2 32	1 95	1 92	98.5
48.2		2.32	1 95	1.92	98.5
49.2		2 35	1 95	1.94	99 5

evaporated to dryness on a hot-plate. After many unsuccessful attempts, including mild muffling starting with a cool muffle furnace, various types of hot-plates, and so forth, we found that creeping and spattering could be avoided if we followed the suggestion of Hald and Eisenman (16), of adding small crystals of analytical reagent sucrose. If the evaporation is carried out at low enough temperature, no spattering occurs. This may be tested easily by holding a piece of white paper just above the crucible. Evaporation took usually from 4 to 6 hours. In extreme cases of creeping, we sometimes resorted to vigorously aspirating the fumes from the crucible.

This is done simply by suspending a piece of small bore glass tubing in the air space inside the crucible, and drawing air through the tube from a water aspirator pump. Several crucibles (in our case ten) can be simultaneously aspirated from a line.

TABLE II
Rat Serum Chlorides

To 0.765 gm. samples of serum 5.98 microequivalents (7.83 microequivalents of Cl per gm. of serum) of Cl were added.

Cl found per gm. serum	Cl recovered per gm. serum	Recovery
<i>microequivalents</i>	<i>microequivalents</i>	<i>per cent</i>
109.4	8.2	104.7
109.1	7.9	100.9
108.9	7.7	98.3
109.9	7.7	98.3
Average 109.3	7.9	100.5

TABLE III
Chloride of Human Blood Serum

To 0.758 gm. samples of serum 57.825 microequivalents of Cl were added.

Cl in sample	Cl recovered	Recovery
<i>microequivalents</i>	<i>microequivalents</i>	<i>per cent</i>
135.19	58.27	100.8
134.80	57.88	100.1
134.95	58.02	100.3
134.75	57.82	100.0
134.66	57.74	99.9
134.67	57.75	99.9
134.70	57.78	99.9
134.76	57.83	100.0
134.67	57.74	99.9
134.55	57.63	99.7
134.76	57.84	100.0
Average 134.77	57.85	100.04

For the determination of sodium and potassium in serum, it is not at all necessary to use platinum crucibles for evaporation of the sulfuric acid. Well glazed porcelain crucibles are satisfactory. This may not be the case in tissue samples.

The dry crucibles are muffled at 500–520° overnight. At this temperature no evaporation of sodium and potassium sulfates occurs, as established

by Broadfoot and Browning (17). At this point, if tissues are being analyzed, phosphate must be removed. This step is unnecessary in the case of serum, as shown by Sobel, Kraus, and Kramer (18). The removal of phosphate from muscle ash is made by the addition of an amount of uranyl nitrate solution in excess of the phosphate present, and the insoluble uranyl phosphate is removed.

For estimation of sodium the following procedure was followed. To the cooled ash are added 5 ml. of dilute HNO_3 (approximately 0.01 N) from a calibrated pipette, and the solution stirred to dissolve all the salts. Since the solution is rich in sodium, two aliquots of 10 per cent or less of the total volume constitute a convenient amount for the sodium determination. A Krogh pipette is used to take these aliquots. We use 0.4 ml. each (8 per

TABLE IV
Muscle Chloride of Rat Diaphragm

Wet weight		Dry weight	Cl per gm. wet weight	Cl per gm. dry weight
mg.		mg.	microequivalents	microequivalents
156.0		36.3	16.7	71.5
152.4		37.1	14.7	60.5
178.4		44.0	14.0	59.9
125.0		29.0	14.1	60.7
185.8		43.9	15.4	65.2
133.4		31.6	15.8	66.8
185.0		43.4	17.3	74.0
144.4		33.5	16.2	69.6
190.8		42.8	16.0	70.9
Average	161.2	37.9	15.6	66.6

cent) on samples of serum ranging between 0.50 and 1.0 gm. The remainder is used for potassium determination (4.2 ml.).

Dry the sodium aliquots and to each add 0.06 to 0.08 ml. of 0.01 N HNO_3 . Swirl to make certain that all the precipitate is in solution. Add 1 ml. of the sodium-precipitating reagent (Reagent 11) and allow the stoppered container to stand for an hour. Long standing will permit evaporation and consequent high results if the containers are unstoppered.

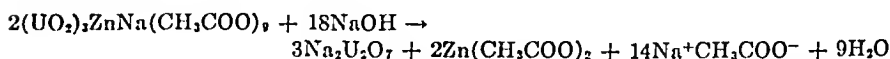
A filter stick is connected to the suction and inserted into the test-tube in which the sodium zinc uranyl acetate was precipitated. Keep it a few mm. below the surface of the liquid. Draw out all the liquid. Lift the filter stick above the level of the precipitate and add 0.5 ml. of the ethanol-acetic acid wash fluid saturated with the triple salt (Reagent 12A). Rotate the test-tube to wash the walls. Lower the filter stick into the liquid and filter off. If any precipitate adheres to the sides of the test-tube,

displace it with the filter stick and wash again with the same wash fluid and filter dry. Wash five times with 0.25 to 0.50 ml. portions of alcohol saturated with sodium zinc uranyl acetate (Reagent 12C). Be certain to wash the walls of the test-tube as well as the outside of the filter stick. Finally wash twice with 0.5 to 1.0 ml. of ether. Push out the asbestos from the filter stick with a clean wire, and wash the filter stick carefully with CO₂-free distilled water. The solution should be clear and a very light yellow. The sodium zinc uranyl acetate may be transferred to an Erlenmeyer flask if necessary. Add 1 drop of phenolphthalein in methanol and titrate to the first permanent pink color with standard CO₂-free NaOH. The volume is important in this end-point, as the precipitate which forms may obscure the color change. In our determinations, we used approximately a 25 ml. volume for the serum sodium determinations, and much less (2 to 5 ml.) for muscle.

TABLE V
Sodium of Human Serum

Weight of serum (3 ml. 0.0100 N NaCl added)	0.0273 N NaOH used in titration	Na found per gm. serum	Na recovered per gm. (39.7 microequiva- lents added)	Recovery
	<i>ml.</i>	<i>microequivalents</i>	<i>microequivalents</i>	<i>per cent</i>
0.7571	3.280	170.4	39.7	100.0
0.7575	3.285	170.6	39.9	100.5
0.7572	3.275	170.2	39.5	99.5
0.7572	3.286	170.7	40.1	100.7
0.7390	3.210	171.0	40.3	101.5
Average . . .		170.6	39.9	100.4

According to Dregus (8) the following reaction is involved in the titration.



Thus each sodium atom is equivalent to 9NaOH, an extremely favorable factor.

Table V indicates the precision of our results. The average of twenty determinations on human serum samples of 0.60 to 0.90 gm. from a pooled stock was 130.7 ± 0.2 microequivalents per gm. (milliequivalents per liter). Duplicate determinations of the same sample do not differ by more than 1 per cent.

Potassium Determination—Potassium is determined in the remaining aliquot. The solution is delivered into a 10 ml. test-tube with a Krogh

pipette and evaporated to dryness. The sides of the tube are washed down and again the solution is evaporated to dryness, in order to confine most of the dried sample to the bottom of the tube. To the dried sulfate add 0.20 ml. of the potassium-precipitating reagent (Reagent 16), 0.20 ml. of Reagent 14, and 2.00 ml. of 95 per cent ethanol. Mix, stopper well, and leave at constant temperature (in our case this was 27°) for 12 hours. All subsequent determinations must be done at this temperature to avoid complications due to temperature corrections for solubility. The added K_2PtCl_6 reagent obviously compensates for the solubility of K_2PtCl_6 in the 80 per cent ethanol. The solubility of this compound is approximately 4 mg. in 100 ml. of solution. For exact determinations a temperature control is essential.

TABLE VI
Human Serum Potassium

To 0.758 gm. samples of serum 4.00 microequivalents of K were added.

Hg(NO ₃) ₂ burette reading	K in sample	K recovered	Recovery
ml.	microequivalents	microequivalents	per cent
1.630	8.89	4.01	100.2
1.620	8.86	3.98	99.5
1.610	8.76	3.88	97.0
1.620	8.86	3.98	99.5
1.640	8.94	4.06	101.5
1.650	9.00	4.12	103.0
1.620	8.86	3.98	99.5
Average. . .	8.88	4.00	100.03

A filter stick is made up as before. In this case the precipitate is fine, and care should be taken that it does not go through the filter stick. The precipitate is washed four times with 0.5 ml. portions of 80 per cent ethanol saturated with K_2PtCl_6 (Reagent 15A), and twice with 0.5 ml. portions of 95 per cent ethanol saturated with K_2PtCl_6 (Reagent 15B). Draw all the liquid off each time. Finally wash twice with 0.5 ml. portions of ether. With a clean wire push out the asbestos from the filter stick and wash the end of the filter stick three times with 1 drop of water. The water held by capillary action in the filter stick is blown out each time. It is preferable to use a small rubber tube for this purpose. Add 0.20 ml. of 0.2 N sodium formate. Wash the sides of the tube down with 1.0 ml. of distilled water. Allow to stand in a boiling water bath for 30 minutes. Filter into a glass vial by means of the micro filtration apparatus described in Fig. 1. Wash three times with 0.2 ml. portions of distilled water. Add 0.3 ml. of 0.2 N HNO_3 , 1 drop of 30 per cent H_2O_2 , and 1 drop of the di-

phenylcarbazone indicator (Reagent 6). Titrate with standard mercuric nitrate (Reagent 7) to the first permanent blue. 3 chloride atoms are equivalent to 1 of potassium.

The average amount of potassium found in ten samples of human serum (same stock used for sodium) was 6.40 microequivalents per gm. of serum. The results ranged from 6.32 to 6.47 microequivalents per gm. Table VI is characteristic of our results. Muscle analyses, because of the high potassium and low sodium, give better results.

DISCUSSION

The mercurimetric method for the determination of chloride is not universally applicable, and we recommend it only under carefully controlled conditions. The titer is dependent on the ionic strength of the solution, volume of the solution, and the hydrogen ion concentration. Strongly oxidizing and reducing ions must be absent. Fortunately such requirements are easily met in these determinations, and we found the mercurimetric method simpler than the more traditional Volhard method, after we established that the two methods gave similar results, with somewhat greater accuracy for the mercurimetric. In our method we used hydrogen peroxide, which resulted in a sharper end-point with diphenylcarbazone indicator. In the case of the micro diffusion determination of chloride hydrogen peroxide presumably oxidizes interfering substances such as sulfites which may diffuse into the KOH with the HCl. In the determination of the chloride liberated from K_2PtCl_6 , the platinum reduced by the action of the formate interferes with the mercurimetric end-point, especially in the presence of hydrogen peroxide; hence the solution is filtered from the platinum as described.

In regard to the sodium determination there are a number of studies of the precipitation of the sodium zinc uranyl acetate, such as the effects of variation of temperature, hydrogen ion concentration of the solution, amount of reagent necessary, and various types of sodium-precipitating reagents such as the substitution of manganese for zinc, interference of other ions, and so forth (*cf.* Broadfoot and Browning (17)). For the accuracy we desire (± 2 per cent), no special precautions are necessary. This also justifies our choice of the use of sodium hydroxide as a method of determination of uranyl ions. The more precise way would be to reduce uranyl ions in a Jones reducer and determine them by titration with oxidizing agents, potentiometric titration (13), or a suitable indicator for determination of the end-point of the titration (*cf.* Kolthoff and Lingane (19)). Potentiometric titration is equally applicable when NaOH is used as titrant. We have made such titrations, using a quinhydrone or glass electrode against saturated calomel, and the results indicate that the

sharpest break in the curve occurred at a pH between 7 and 8. Therefore phenolphthalein is a suitable indicator. This pH agrees well with the calculated value. If the pH at the end-point is caused by the sodium acetate ion concentration, which is of the order of 2×10^{-3} , then the hydrogen ion concentration is

$$(H^+) = \sqrt{\frac{K_w \times K_{\text{acid}}}{\text{concentration}}} = \sqrt{\frac{10^{-14} - (2 \times 10^{-3})}{2 \times 10^{-3}}} = 10^{-8}$$

or pH = 8 (approximately).

Cresol red, owing to its yellow color, is unsatisfactory, since the sodium zinc uranyl acetate solution is also yellow. Obviously the potentiometric titration would afford an excellent method for the determination of the end-point. We present the indicator end-point method as the more available method to most workers.

In the potassium determination we preferred to compensate for the solubility of K_2PtCl_6 by adding the amount of this salt which would be lost, rather than the usual method of drying the precipitate and adding 80 per cent ethanol saturated with K_2PtCl_6 , since the Na_2PtCl_6 does not go into solution readily. This was observed by Cunningham, Kirk, and Brooks (7), who suggested the dried chlorides of sodium and potassium be taken up in 80 per cent ethanol saturated with K_2PtCl_6 . Unfortunately the sulfates of sodium and potassium are rather insoluble in 80 per cent ethanol, and the present method described was resorted to. This is, however, not without advantage, since if there is an excess of Na_2SO_4 above the H_2PtCl_6 , it does not interfere with the further chloride determination. The results of our determinations, and also those of Cunningham, Kirk, and Brooks, would be high if a large excess of H_2PtCl_6 -precipitating reagent were used, owing to the solubility product effect, and we recommend the use of the smallest possible volumes.

SUMMARY

A relatively inexpensive method of analysis involving titrimetric procedures is described for the simultaneous determination of sodium, potassium, and chloride on the same sample of biological fluid or tissue, with a precision of 1 to 2 per cent on samples of the order of 20 to 1000 mg. Chloride is driven out of the sample as hydrochloric acid by digestion in a closed system with strong sulfuric acid, and collected in strong alkali, after which it is determined by a mercurimetric titration which gives a color change from colorless to blue. Sodium is precipitated as the sodium zinc uranyl acetate, the acetate of which is titrated with standard alkali to a phenolphthalein end-point, each sodium atom being equivalent to 9 atoms of alkali. Potassium is precipitated as potassium chloroplatinate

which is reduced with formate, and the liberated chloride is determined by mercurimetric titration, 3 chloride atoms being equivalent to 1 of potassium.

Examples of analyses of sodium, potassium, and chloride of human serum are given; and of rat serum chloride, and muscle chloride of the rat diaphragm.

BIBLIOGRAPHY

1. Joseph, N. R., and Stadie, W. C., *J. Biol. Chem.*, **125**, 795 (1938).
2. Oster, R. H., *J. Biol. Chem.*, **131**, 13 (1939).
3. Oster, R. H., and Amberson, W. R., *J. Biol. Chem.*, **131**, 19 (1939).
4. Dean, R. B., and Fishman, M. M., *J. Biol. Chem.*, **140**, 807 (1941).
5. Dubsky, J. V., and Trtilek, J., *Mikrochemie*, **6**, 315 (1933).
6. Schales, O., and Schales, S. S., *J. Biol. Chem.*, **140**, 879 (1941).
7. Cunningham, B., Kirk, P. L., and Brooks, S. C., *J. Biol. Chem.*, **139**, 21 (1941).
8. Dregus, M., *Biochem. Z.*, **303**, 69 (1939).
9. Barber, H. H., and Kolthoff, I. M., *J. Am. Chem. Soc.*, **50**, 1625 (1931).
10. Kolthoff, I. M., and Sandell, E. B., *Textbook of quantitative inorganic analysis*, New York, 521 ff. (1937).
11. Emich, F., translated by Schneider, F., *Microchemical laboratory manual*, New York (1932).
12. Trevan, J. W., *Biochem. J.*, **19**, 1111 (1925).
13. Kryukov, P. A., and Kalarova, E. D., *Pedology*, U. S. S. R., No. 12, 76 (1939).
14. Scholander, P. F., *Science*, **95**, 177 (1942).
15. Krogh, A., and Keys, A., *J. Chem. Soc.*, 2436 (1931).
16. Hald, P. M., and Eisenman, A. J., *J. Biol. Chem.*, **118**, 275 (1937).
17. Broadfoot, W. M., and Browning, G. M., *J. Assn. Off. Agric. Chem.*, **24**, 916 (1941).
18. Sobel, A. E., Kraus, G., and Kramer, B., *J. Biol. Chem.*, **140**, 501 (1941).
19. Kolthoff, I. M., and Lingane, J. J., *J. Am. Chem. Soc.*, **55**, 1872 (1933).

THE HYDROLYSIS OF BIOTIN SULFONE*

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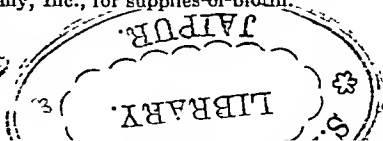
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During the course of studies on the structure of biotin, $C_{10}H_{16}O_3N_2S$, we attempted to prepare the sulfone of the diaminocarboxylic acid which we had obtained from biotin by hydrolysis with $Ba(OH)_2$ (1). Oxidation of the sulfate of the diaminocarboxylic acid directly with H_2O_2 did not yield the sulfone. Furthermore, treatment of biotin sulfone with $Ba(OH)_2$ under conditions which were known to bring about the hydrolysis of the urea linkage of biotin itself did not yield the desired compound. We therefore undertook to prepare the compound by the oxidation of the diacetyl derivative of the diaminocarboxylic acid to the diacetyldiaminocarboxylic acid sulfone, followed by the hydrolysis of the acetyl groups with acid.

The diaminocarboxylic acid from biotin was acetylated in good yield with acetic anhydride and $NaOH$. Oxidation of the diacetyl derivative with H_2O_2 in glacial acetic acid gave the sulfone of the diacetyldiaminocarboxylic acid. The acetyl groups were removed by hydrolysis with concentrated HCl at 120° for 1 hour. From the hydrolysis mixture we obtained a crystalline, slightly hygroscopic hydrochloride, m.p. $142-152^\circ$, which could be best recrystallized from concentrated HCl .

Kögl and de Man (2) have stated that hydrolysis of biotin sulfone with concentrated HCl at 200° for $\frac{1}{2}$ hour yields a 9-carbon diaminocarboxysulfonic acid. These workers did not obtain their hydrolysis product in a pure state, but characterized the compound by an N analysis on the picrolonate, by C, H, N, and S analyses on the dilituric acid derivative, and by a titration curve on the hydrochloride of the crude material. It seemed possible to us that the same compound might have been formed by our treatment of the sulfone of the diacetyldiaminocarboxylic acid with concentrated HCl at 120° . For comparison, therefore, we carried out the hydrolysis of biotin sulfone under the conditions described by Kögl and de Man. By this treatment of biotin sulfone with concentrated HCl at 200° for $\frac{1}{2}$ hour we were able to obtain a crystalline product which after

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two crystallizations from concentrated HCl was obtained in excellent yield. The same compound was obtained in only slightly lower yield when the hydrolysis was carried out for $1\frac{1}{2}$ hours. The compound appeared to be identical in all its properties with the compound we had also obtained by hydrolysis of the sulfone of the diacetyldiaminocarboxylic acid with HCl at 120° . Furthermore the dilituric acid derivatives prepared from both compounds appeared to be identical with one another and possessed the melting point ($235\text{--}240^\circ$) reported by Kögl and de Man for the diliturate of the hydrolysis product described by them.

It seemed likely, therefore, that the hydrolysis product which we had obtained was the same one obtained by Kögl and de Man. However, the analytical values we obtained on this compound agreed more closely with those for the dihydrochloride of the diaminocarboxylic acid sulfone than for the dihydrochloride of the diaminocarboxysulfonic acid, and raised the question whether Kögl and de Man had obtained a sulfonic acid. Conclusive proof that the compound under consideration was the diaminocarboxylic acid sulfone rather than a sulfonic acid would be provided if the substance could be reconverted to biotin sulfone by the action of phosgene, a type of reaction which has been shown to occur in excellent yield in the resynthesis of biotin from the diaminocarboxylic acid (3).

By treatment of the compound with phosgene in Na_2CO_3 solution at 0° we were able to obtain biotin sulfone, m.p. $265\text{--}275^\circ$,¹ in 95 per cent yield. Recrystallization of this product gave pure biotin sulfone, m.p. $273\text{--}275^\circ$, which was identical in all its properties with biotin sulfone obtained by oxidation of biotin with H_2O_2 .

EXPERIMENTAL

Diacetyldiaminocarboxylic Acid—30 mg. of the diaminocarboxylic acid sulfate, prepared from biotin by hydrolysis with $\text{Ba}(\text{OH})_2$ (1), were dissolved in 1 cc. of water and the solution was cooled in an ice bath and made alkaline to phenolphthalein with a few drops of 4 N NaOH. There were then added in four portions 75 mg. of acetic anhydride and enough 4 N NaOH to keep the solution alkaline to phenolphthalein. The solution was then acidified to Congo red with 3 N HCl and was concentrated to dryness *in vacuo*. The dry residues were extracted four times with 5 cc. of hot chloroform and the combined chloroform extracts were evaporated *in vacuo*. Crystallization of the residue from a few drops of methanol yielded 25 mg. of prisms, m.p. $184\text{--}186^\circ$. For analysis a portion of this material was sublimed at 170° and 10^{-5} mm. The sublimate melted at $187\text{--}189^\circ$.

$\text{C}_{11}\text{H}_{12}\text{O}_4\text{N}_2\text{S}$ (302.4). Calculated, C 51.63, H 7.34; found, C 51.95, H 7.69

¹ The melting points reported in this paper are micro melting points.

Diacetyldiaminocarboxylic Acid Sulfone—For the preparation of the sulfone 20 mg. of the diacetyldiaminocarboxylic acid were dissolved in 2 cc. of glacial acetic acid and to the solution was added 0.4 cc. of 30 per cent H_2O_2 . The solution was kept at room temperature for 16 hours and then evaporated to dryness *in vacuo*. The crystalline residue was recrystallized from a mixture of methanol and ether, and yielded 20 mg. of needles, m.p. 209–211°.

$\text{C}_{11}\text{H}_{22}\text{O}_4\text{N}_2\text{S}$.	Calculated	C 46.69, H 6.63, S 9.59
(334.4)	Found.	" 46.43, " 6.86, " 9.55

Hydrolysis of Diacetyldiaminocarboxylic Acid Sulfone—As a preliminary experiment to determine a suitable method of hydrolysis of the diacetyldiaminocarboxylic acid sulfone 1 mg. of the compound was dissolved in 0.5 cc. of concentrated HCl and heated 1 hour at 120°. The solution was concentrated to dryness *in vacuo*. The semicrystalline residue was dissolved in 0.2 cc. of 33 per cent acetic acid and 0.1 cc. of this solution was used for a micro-Van Slyke amino nitrogen determination in the Warburg apparatus.

$\text{C}_{11}\text{H}_{22}\text{O}_4\text{N}_2\text{S}$ (334.4). Calculated, $\text{NH}_2\text{-N}$ 8.38; found, $\text{NH}_2\text{-N}$ 8.04

20 mg. of the diacetyldiaminocarboxylic acid sulfone were dissolved in 2 cc. of concentrated HCl and the solution was heated at 120° for 1 hour. The solution was filtered and was concentrated to dryness *in vacuo*. Attempts to crystallize the residue from water solutions resulted in semicrystalline material. The residue was dissolved in a few drops of hot concentrated HCl. The crystals obtained from the cooled solution were washed twice with a few drops of ethanol. The material was recrystallized from concentrated HCl and the crystals were washed with ethanol and were dried. 11.8 mg. of crystals in the form of elongated plates were obtained. The melting point range, 142–152°, was not changed by recrystallization. A qualitative test showed sulfur to be present.

The dilituric acid derivative was prepared as follows: 1 mg. of the hydrolysis product was dissolved in a few drops of water, and to this was added a saturated aqueous solution of dilituric acid (5-nitrobarbituric acid) in excess. The crystalline white precipitate which formed was washed with water, and was recrystallized from water in the presence of a drop of dilituric acid solution. The recrystallized compound melted with decomposition at 235–240°.

Hydrolysis of Biotin Sulfone—50 mg. of biotin were oxidized to biotin sulfone with H_2O_2 (1). After recrystallization 49.7 mg. of pure biotin sulfone were obtained. The 49.7 mg. of sulfone were divided in two equal portions and each was dissolved in 5 cc. of concentrated HCl. The solu-

tions were sealed in heavy walled tubes and the tubes were placed in an oil bath, held at 200° , for $\frac{1}{2}$ hour. The slightly yellow reaction mixtures were combined and were concentrated to dryness. The residue was dissolved in a small amount of water, was filtered, and was concentrated to dryness. The residue was dissolved in a few drops of hot concentrated HCl and was allowed to cool. The crystals which formed were washed twice with a few drops of ethanol and were recrystallized by the same procedure. The resulting material, 48.7 mg. of colorless, elongated plates, melted at $142-152^{\circ}$. Further crystallization did not change the melting point range. A similar experiment in which 20 mg. of biotin sulfone were treated with 4 cc. of concentrated HCl at 200° for $1\frac{1}{2}$ hours yielded 15 mg. of the hydrolysis product, m.p. $142-152^{\circ}$. A qualitative test showed that sulfur was present in the compound.

$C_8H_{10}O_4N_2SCl_2$	Calculated.	C 33.44, H 6.24, N 8.66, Cl 21.94
(323.2)	Found.	" 33.20, " 6.53, " 8.40, " 21.44

5.0 mg. of the recrystallized hydrolysis product were used for the preparation of the diliturate by the procedure already described. 8.7 mg. of the derivative were obtained, which on recrystallization yielded 6.3 mg. The compound melted with decomposition at $235-240^{\circ}$.

Treatment of Hydrolysis Product with Phosgene—20 mg. of the recrystallized hydrolysis product of biotin sulfone were dissolved in 2 cc. of cold 5 per cent Na_2CO_3 solution. The solution was cooled in an ice bath while phosgene was passed in until the solution was acid to Congo red. A white crystalline solid separated from the acid solution. The mixture was concentrated to a small volume and then heated to dissolve the solid. Long needles separated from the cooled solution. The crystals were washed twice with water and were dried. 16.4 mg. of needles, m.p. $265-275^{\circ}$, were obtained. By concentration of the mother liquors to dryness and by extraction of the dry residue with hot glacial acetic acid, an additional 1.5 mg. of crystals, m.p. $265-275^{\circ}$, were obtained. The combined crystalline fractions were recrystallized from water and yielded 15.5 mg. of pure biotin sulfone, m.p. $273-275^{\circ}$. A mixed melting point with biotin sulfone showed no depression.

$C_{10}H_{16}O_5N_2S$	Calculated.	C 43.47, H 5.84, N 10.13
(276.3)	Found.	" 43.61, " 6.02, " 10.07

The authors wish to express their appreciation to Dr. Julian R. Rachele of this laboratory for carrying out the microanalyses.

SUMMARY

Treatment of biotin sulfone with concentrated HCl at 200° for $\frac{1}{2}$ hour hydrolyzes the urea linkage and gives the dihydrochloride of the diamino-

carboxylic acid sulfone in good yield. This appears to be the same compound obtained by Kögl and de Man under these conditions, but which was believed by them to be a 9-carbon sulfonic acid, and was used as proof for the presence of a sulfur-containing ring in biotin. The same compound is obtained by hydrolysis of the diacetyl derivative of the diaminocarboxylic acid sulfone with concentrated HCl at 120°. Additional and conclusive proof of the structure of the hydrolysis product was furnished by the re-synthesis of biotin sulfone on treatment of the compound with phosgene.

BIBLIOGRAPHY

1. Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **141**, 207 (1941).
2. Kögl, F., and de Man, T. J., *Z. physiol. Chem.*, **269**, 81 (1941).
3. Melville, D. B., Hofmann, K., and du Vigneaud, V., *Science*, **94**, 308 (1941).

STUDIES ON HEMORRHAGIC ANEMIA IN DOGS*

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Anemia studies have been carried on in this laboratory for several years in dogs on whole milk rations supplemented in various ways. The anemias have been induced by nutritional and hemorrhagic methods (1, 2). Nutritional anemia in puppies receiving such a ration was found curable by the addition of inorganic iron and copper salts, although regeneration was suboptimal in a number of cases (1). When anemia was produced in adult dogs by mild phlebotomy, the response to these mineral supplements was usually good (3), although occasionally it was slow and erratic and under more severe phlebotomy recovery might not occur at all. The most consistent failure in blood regeneration occurred when a high level of cobalt was added to the ration (4). The addition of liver extract to these rations resulted in rapid remission from the anemias in all cases. The behavior of dogs is thus comparable to that of rats receiving milk rations. In the latter species iron and copper supplements produce and maintain a normal blood stream but under the strain of hemorrhage evidence indicates that liver supplements furnish additional factors not adequately supplied by the mineralized milk alone (5). It is the purpose of this paper to report investigations into the nature of the substance or substances in liver extract responsible for this stimulation of blood formation.

Recently considerable progress has been made in the clarification of the problems of dog nutrition and it has been possible for us to obtain normal growth in dogs for a considerable period of time on a highly purified ration supplemented only with synthetic vitamins (6). Phlebotomy and blood regeneration studies on this ration have been most informative and will also be presented in this paper.

EXPERIMENTAL

Hemorrhagic Anemia Studies on Mineralized Milk Diet

Adult mongrel dogs were given whole milk twice daily *ad libitum*. They were bled weekly from the external jugular vein in amounts usually ranging

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from 20 to 25 per cent of their total blood. After 4 to 8 weeks a rather stable state of anemia was reached at a hemoglobin level of 7 to 9 gm. per cent and phlebotomy was discontinued. 20 mg. of cobalt as cobaltous chloride were then added daily to the ration and this addition continued for 1 to 2 weeks followed by 30 mg. of iron as ferric chloride and 3 mg. of each of copper and manganese as cupric and manganous sulfates. After 1 to 2 weeks, during which time the hemoglobin level of the blood increased very little, the liver supplement extract to be tested was given for a period of 2 weeks. Active liver preparations were found to effect a rise in blood hemoglobin to levels of 13 to 14 gm. per cent in this time.

In addition to the hemoglobin determination, red cell counts and hematocrit determinations were made routinely and later a plasma iron analysis was also performed routinely. Blood samples for analysis were always taken from the radial vein. An 18 cc. sample was drawn into a 30 cc. syringe, the dead space of the syringe being filled with Wintrobe's non-shrinking oxalate solution. Hemoglobin determinations were made by the *method of Evelyn (7), and hematocrit determinations with Wintrobe tubes*. The plasma iron determinations were made on filtrates of blood plasma from a hot trichloroacetic acid precipitation. The filtrates were treated with thioglycolic acid and the ferrous iron then reacted with α, α' -bipyridine (pH 5.4). The resulting color was determined on the Evelyn photoelectric colorimeter. This method is to be published in detail in the near future.

The data from the analysis of blood drawn during the assay of various liver preparations and nutrient materials are given in Table I. For the sake of brevity only the initial and final hemoglobin levels and the initial and maximum plasma iron levels are given. In evaluation of the hemoglobin response to the various supplements it was necessary to minimize errors arising from fluctuations in weight. Assuming 8 per cent of body weight as blood, the total body hemoglobin was calculated from the hemoglobin level of the blood. These values, together with the known amount of hemoglobin removed as the analysis sample, permitted the calculation of the total "hemoglobin made" as given in the last column.

The typical blood picture as seen in the anemia and in the course of the remission is shown in Fig. 1. The anemia is characterized by a fairly high erythrocyte count, extremely small cells, low hematocrit, low hemoglobin, and low plasma iron. The administration of liver extract results in striking increases in hemoglobin, hematocrit, erythrocyte count, and plasma iron but the mean erythrocyte volume and saturation index remain practically unchanged.

The possibility of iron deficiency as suggested by the low mean cell volume and plasma iron values led us to investigate first the question of whether or not the ferric iron as fed was being properly reduced and ab-

TABLE I

Effect of Various Supplements on Blood Picture of Phlebotomized Dogs Receiving the Mineralized Milk Ration

Assay No.	Dog No.	Daily supplement	Days	Initial Hb	Final Hb	Initial plasma Fe	Plasma Fe maximum	Hb made
				gm. per cent	gm. per cent	γ per cent	γ per cent	gm.
1	23	10 mg. pyridoxine hydrochloride	27	7.7	8.5			14
2	23	25 gm. liver extract powder 1:20	15	8.5	14.0			60
3	23	FeCl ₂ equivalent to 100 mg. iron. 200 mg. ascorbic acid	13	7.4	9.1	65	73	17
4	23	Lead filtrate equivalent to 25 gm. liver extract powder 1:20	15	9.1	11.4	73	328	23
5	23	12 gm. solubilized liver extract	16	11.4	11.7	328	228	6
6	23	20 " Fraction D	7	11.7	13.1	228	179	13
7	23	Lead ppt. equivalent to 30 gm. Fraction D	12	7.3	8.4	36	38	10
8	24	50 gm. whole dry liver	14	8.1	13.6			55
9	24	1.35 gm. ascorbic acid intravenously over period	10	5.9	6.7			0
10	24	52 mg. nicotinic acid, 6.3 mg. riboflavin, 10.5 mg. thiamine chloride, 1 mg. pyridoxine HCl, 12.6 mg. Ca pantothenate, 250 mg. choline chloride	16	6.5	7.3	24	26	13
11	24	FeCl ₂ equivalent to 60 mg. iron	20	7.3	10.8	26	23	41
12	24	Ash equivalent to 25 gm. liver extract powder 1:20	14	8.1	9.4	25	30	12
13	24	20 gm. liver extract powder 1:20	14	9.4	13.5	30	296	34
14	24	2 cc. (4 units) Lilly pernicious anemia concentrate intravenously	14	6.6	8.7	11	39	21
15	24	Lead and mercury filtrate equivalent to 30 gm. Fraction D	15	8.6	10.2	53	40	17
16	24	Mercury ppt. of lead filtrate equivalent to 30 gm. Fraction D	13	10.2	12.3	40	74	23
17	24	Uropterin concentrate equivalent to 4 liters human urine orally	7	7.0	8.2	13	22	13
18	24	Uropterin concentrate equivalent to 4 liters human urine intravenously	14	8.5	10.7	30	134	20
19	24	2-3 gm. ox bile salts	15	10.7	11.7	134	50	15
20	24	25 gm. liver extract powder 1:20	14	11.7	13.3		102	26
21	24	3 gm. hog bile salts, 0.52 gm. cysteine HCl, 1 mg. iodine as KI, 1.1 mg. thiamine chloride and riboflavin, 22 mg. nicotinic acid, 0.66 mg. pyridoxine HCl, 5.5 mg. Ca pantothenate, 0.7 gm. choline chloride, 0.38 gm. haliver oil (Abbott's), 100 mg. ferrous iron as Fe(NH ₄) ₂ (SO ₄) ₂	13	8.0	11.2	30	31	31

TABLE I—*Concluded*

Assay No	Dog No	Daily supplement	Days	Initial Hb	Final Hb	Initial plasma Fe	Plasma Fe maximum	Hb made
				gm per cent	gm per cent	γ per cent	γ per cent	gm
22	13	FeCl ₂ equivalent to 24 mg iron intravenously (12 injections)	20	8 4	9 7	43	757	26
23	13	FeCl ₂ equivalent to 80 mg. iron	20	9 7	11 6	60	81	31
24	13	15 gm liver extract powder 1:20	21	11 6	15 0	81	216	63
25	13	1 mg thiamine chloride, 6 mg riboflavin, 60 mg nicotinic acid, 1 5 mg pyridoxine HCl, 500 mg. choline chloride, 13 3 mg. Ca pantothenate, 500 mg inositol, 500 mg l-asparagine	20	7 3	9 1	57	72	30
26	13	25 gm liver extract powder 1:20	14	9 1	12 5	62	273	48
27	13	Lead and mercury filtrate equivalent to 30 gm. Fraction D	15	9 5	12 4	55	126	51
28	13	Mercury ppt. of lead filtrate equivalent to 30 gm Fraction D	16	8 3	9 4	49	81	18
29	13	Lead and mercury filtrate equivalent to 30 gm Fraction D	14	9 4	10 7	81	163	22
30	13	25 gm Fraction D	14	10 7	12 3	163	167	22
31	13	Uropterin concentrate equivalent to 100 gm whole dry liver	16	9 5	11 4	65	103	28
32	13	50 gm whole dry liver first 16 days	30	11 4	14 5	71	131	54
33	13	500 mg l-cystine	7	7 9	7 9	11	35	-2
34	13	520 " cysteine hydrochloride	17	8 2	10 4			28
35	13	3 gm hog bile salts, 500 mg cysteine HCl, 1 mg. iodine as KI, 1 4 mg thiamine chloride and riboflavin, 28 mg nicotinic acid, 0 85 mg pyridoxine HCl, 7 1 mg. Ca pantothenate, 0 57 gm choline chloride, 0 38 gm haliver oil (Abbott's), 100 mg ferrous iron as Fe(NH ₄) ₂ (SO ₄) ₂	14	9 9	12 3	75	99	41
36	27	15 gm. Fraction D (13 days), 25 gm liver extract powder 1 20 (7 days)	20	8 4	12 6	51	106	37
37	27	300 mg. p-aminobenzoic acid	14	7 8	8 6			14
38	29	FeCl ₂ equivalent to 80 mg. iron, 200 mg ascorbic acid	19	7 7	10 7	142	41	28
39	3	25 gm Fraction D	15	8 4	12 0	96	160	45
40	3	5 gm ox bile salts	7	11 0	11 8	62	87	13
41	3	4 5 gm hog bile salts	15	11 8	13 6	87	53	30

sorbed. A number of experiments were carried out in an attempt to answer this question. Ferrous iron was substituted for ferric iron and increased amounts were fed alone and with a reducing agent, ascorbic acid

(Assays 3, 11, 23, 38, Table I). Ferrous iron was injected intravenously to the level of toxicity symptoms (Assay 22). The response in all cases was far inferior to that given by liver preparations and it was concluded that iron deficiency was not the primary cause of remission failure. That it was a complicating secondary factor seemed altogether probable and so ferrous iron was substituted for ferric iron and thereafter the mineral supplements were supplied continually during the bleeding, anemic, and remission periods.

The organic nature of the factor in liver is evident from the failure of the ash fraction of liver extract to produce remission (Assay 12). When the original liver extract was given, a satisfactory response occurred (Assay 13).

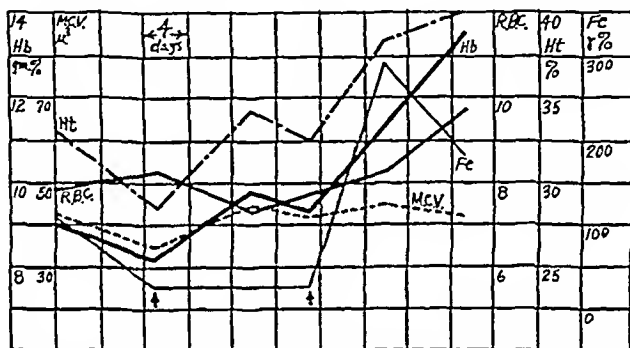


FIG. 1. Effect of equivalent amounts of liver extract ash and liver extract powder on the blood picture of Dog 24 receiving the mineralized milk ration with cobalt. The supplements were initiated at the points indicated by arrows. Hb, blood hemoglobin in gm. per cent; r.b.c., erythrocyte count in millions per c.mm.; m.c.v., mean erythrocyte volume in cu. micra; Fe, plasma iron in mg. per cent; Ht, hematocrit per cent.

The low mean cell volume also suggested a possible deficiency of pyridoxine, since this deficiency anemia is also microcytic. Supplements of vitamin B₆ far in excess of the amount present in the curative dose of liver extract failed to produce improvement (Assay 1). Pyridoxine in supplement mixtures with thiamine, riboflavin, nicotinic acid, calcium pantothenate, choline, asparagine, and inositol produced slight to partial remissions but not at all comparable to those produced by liver extracts (Assays 10 and 25). The slight activity of the water-soluble vitamin mixtures is of interest, since several members of this group have been associated with hematopoiesis. Chronic thiamine deficiency in man has been reported by Mason and Mason (8) to be accompanied by a macrocytic hyperchromic anemia, although this was not observed in acute thiamine deficiency. Gobell (9)

obtained increases in the red blood cell count of premature anemic infants by the administration of nicotinamide and riboflavin. Dollchen (10) reported hemoglobin and erythrocyte responses to pyridoxine, nicotinamide, and riboflavin in a study of hemorrhagic anemia in rabbits. György *et al.* (11) observed some hemoglobin increase after the administration of riboflavin to phlebotomized dogs. The action of choline is of considerable interest since the observation of Davis (12) that choline is the substance in liver responsible for the depressing effect on cobalt polycythemia. Since a high level of cobalt was used in this ration and may be wholly or in part responsible for remission failure, the action of an antagonizing substance such as choline would deserve investigation. Whatever the rôle of these water-soluble vitamins in hematopoiesis in dogs may be, it is apparent that they are not of primary importance under the conditions of this study.

A similar neutralizing effect on cobalt toxicity has been attributed to vitamin C by Barron and Barron (13). These investigators observed that intravenous injections of vitamin C would prevent polycythemia in rabbits if given simultaneously with cobalt. No effective antagonistic action was obtained with vitamin C in our studies if one may use blood regeneration as the criterion for this action (Assays 3, 9, and 38). The low vitamin C content of active liver preparations also argues against the possibility that this vitamin is the active constituent of liver extract.

A cysteine-cobalt complex has been investigated for its biological implications by Griffith *et al.* (14). They found that acute cobalt toxicity in rats could be prevented by the addition of cysteine or cystine to the diet. In our studies 500 mg. per day of cystine were ineffective over a 7 day period (Assay 33). Cysteine hydrochloride, however, fed at a level of 520 mg. per day showed considerable promise of activity (Assay 34). Since it is cysteine and not cystine which forms the complex with cobalt, the negative result with the latter suggests a failure by the dog to reduce this amino acid to cysteine. At all events, it appears that at least a part of the activity of liver extracts may be attributed to their content of cysteine or small molecular weight peptides containing cysteine, as for example, glutathione. Absorbed cobalt may then be tied into the cobalt-cysteinic complex which is incapable of exerting the toxicological actions produced by cobalt alone.

The rôle of bile salts in the absorption of various substances essential to blood formation has been widely investigated. Scyderhelm *et al.* (15, 16) found that bile fistula dogs developed an anemia which was autoregulated at about two-thirds the normal hemoglobin level. The anemia was preventable or curable by feeding bile, bile acids, and finally by light-activated ergosterol. Takasu (17) repeated this study of "acholic cachexia" in dogs and found a similar blood picture. He observed, in turn, that bile acids were effective if vitamin D were present. The relation of dietary calcium,

phosphorus, and vitamin D to iron absorption has been investigated (18). In general an optimum calcium-phosphorus ratio for ossification with adequate vitamin D establishes optimum conditions for iron absorption. Conceivably these conditions prevent an excess of phosphate in the intestinal tract and a minimum of dietary iron is made unavailable as insoluble ferric phosphate. Mild anemias may then arise from vitamin D deficiency. Josephs (19) found that iron absorption was lowered in infants receiving a diet deficient in vitamin D.

Bile salts may also play a rôle in iron absorption independent of their relation with the fat-soluble vitamins. Hawkins *et al.* (20) observed a decreased hemoglobin production in bile fistula dogs which could not be entirely accounted for by the decrease in iron absorption. Smith and Crandall (21) found that bile fistula dogs remain persistently anemic in spite of an unimpaired iron absorption as evident from their plasma iron studies.

These relationships suggested the trial of bile salts¹ in our experimental anemia. In three trials, two may be considered as indicative of definite activity (Assays 40 and 41); the other showed considerably less stimulation (Assay 19). Although the plasma iron studies in these assays are incomplete, the available data show no elevation of plasma iron as might be expected if the responses obtained were due to a better absorption of iron. Although none of the responses was comparable to those obtained with liver extracts; nevertheless, it seems apparent that part of the activity of the liver preparations may be attributed to their content of bile salts.

The rôle of xanthopterin or uropterin in blood formation was first suggested by the work of Tschesche and Wolf (22). These investigators found that uropterin produced striking increases in the erythrocyte count of young rats made anemic by being fed goat milk. Simmons and Norris (23) working with a nutritional anemia in fingerling Chinook salmon found that intraperitoneal injection of liver extract would produce rapid red blood cell count increases. The active principle of liver was found to be uropterin.

On the basis of the above findings it was decided to try uropterin in this study. No synthesis of the pigment was attempted. Instead, concentrates of it were prepared from liver and human urine according to the concentration methods of Koschara (24, 25). A preparation made from whole dry liver was fed at a level equivalent to 100 gm. of the original liver per day (Assay 31). One preparation from urine equivalent to 4 liters of urine per day was fed orally (Assay 17), while a more purified preparation was injected intravenously (Assay 18) at the same level. All three preparations showed appreciable activity but were considerably below the respon-

¹ Suggested by Dr. David Klein of The Wilson Laboratories, Chicago, Illinois.

ses elicited by liver extract. These preparations contained far more uropterin than would be present in the curative dose of liver extract. The rôle of uropterin, then, seems doubtful, although its deficiency may be at least in part responsible for the failure of blood regeneration.

An attempt at fractionation of the active liver extracts "Fraction D" and "liver concentrate powder-1:20" (The Wilson Laboratories) was made by use of heavy metal precipitation. In both cases aqueous solutions of the liver extracts were adjusted alkaline to phenolphthalein with NH_4OH and sufficient 30 per cent basic lead acetate solution added to insure complete precipitation.

The basic lead acetate precipitate was suspended in water and H_2S gas bubbled through until all the lead salts were converted into their corresponding acids and the lead precipitated as sulfide. This process was repeated and the combined filtrates neutralized and concentrated under reduced pressure. This preparation was designated as the "lead precipitate" and was fed at a level of 30 gm. equivalent of the original Fraction D (Assay 7). The relative inactivity of this preparation can be seen from the character of the hemoglobin and plasma iron changes.

The basic lead acetate filtrate obtained was treated with H_2S gas until the excess lead was precipitated. The lead sulfide was then separated by filtration, washed with hot distilled water, and the filtrate and washings neutralized and concentrated under reduced pressure. This preparation designated as the "lead filtrate" was used in Assay 4. Considerable activity was disclosed but the preparation was so difficult to feed at therapeutic levels that it was necessary to work with more purified concentrates.

The lead filtrate was therefore used for the preparation of two mercury fractions. The lead filtrate was adjusted to pH 6.8 and warm saturated HgCl_2 solution added and the precipitate formed was filtered off. The filtrate was again adjusted to pH 6.8 and more HgCl_2 solution added until complete precipitation was obtained. Excess mercuric ion was removed with H_2S and the mercuric sulfide precipitate was washed with warm water. The combined filtrate and washings were concentrated under reduced pressure and enough ethanol added to precipitate the inorganic salts. The aqueous ethanol-soluble material was filtered off, neutralized, and the alcohol removed by distillation under reduced pressure. This preparation was designated as the "lead and mercury filtrate" and was used in Assays 15, 27, and 29. Activity was evident in all three trials but in only one (Assay 27) was it equivalent to that of whole liver extract. The other two trials showed considerably less activity.

The mercury precipitate fraction from the lead filtrate was suspended in water and H_2S gas passed in until no more HgS was formed. The HgS was washed with boiling water and the filtrate and washings neutralized

with KOH (Congo red). More H_2S was passed in and the precipitate was allowed to settle. This second HgS precipitate was washed with hot water and the combined filtrate and washings neutralized and concentrated under reduced pressure. This preparation was designated as the "mercury precipitate of the lead filtrate" and was used in Assays 16 and 28. Slight to moderate activity was observed in these two trials but the preparation seemed somewhat inferior to the mercury filtrate of the lead filtrate.

From the results obtained it seems apparent that the principal regenerating activity in liver extract is not precipitated by basic lead acetate. More activity seems to pass into the mercury filtrate than into the precipitate, but the failure of the complete activity of liver extract to stay in any one fraction indicates that more than one active substance is involved. The possibility that none of these fractions would ever show its true activity must be considered when the actual mechanical difficulties of feeding these preparations are taken into account. The loss of the taste-masking substances as precipitated by basic lead acetate leaves an extremely bitter preparation containing the activity. Nausea and even vomiting sometimes accompanied the feeding of this lead filtrate and its two mercury fractions. Optimum blood formation might not be expected to occur under such adverse conditions of assay.

The activity of the lead filtrate fraction suggested the trial of antipernicious anemia concentrates, since this substance is not precipitated by basic lead acetate. A preparation obtained from Eli Lilly and Company containing 2 units per cc. was injected intravenously (Assay 14). The activity was only moderate as compared to that of the whole liver extracts and it was concluded that this factor was not involved. The excellent regenerating activity of liver Fraction D, which is very low in the antipernicious anemia principle, is better evidence for this conclusion.

The suggestion from the assay of various liver fractions that more than one substance is concerned in blood regeneration under these conditions as well as the activity of the several pure substances recounted above suggested the trial of a mixture of all of these active substances. Activity comparable to that of liver extract was obtained by a mixture of the synthetic B vitamins, hog bile salts, cysteine, increased iron, iodine, and haliver oil (Assays 21 and 35). The poor physical condition of the dogs used for assay after many months of steady experimentation lends considerable weight to the regeneration observed. Especially noteworthy was the anorexia and marked loss of weight of Dog 13 just prior to Assay 35. This loss of 3 kilos of body weight was far greater than that usually encountered in our assay work and indicated a severe deficiency or toxicity. The supplement mixture afforded the production of a relatively normal mean erythrocyte volume in Dog 13 but in neither assay were the plasma iron levels

increased appreciably as is the case when cure is effected with liver extracts. It is possible that yet another substance in liver controls the level of iron in the plasma and perhaps its form or state. The general conclusions which may be arrived at from these studies on the milk ration will be considered after the work on the synthetic ration has been discussed.

Anemia Studies on Synthetic Ration

In our studies on the vitamin B complex in the nutrition of the dog we have used a highly purified sucrose-casein ration supplemented with synthetic vitamins and liver extracts (26, 27). We have found that if such a ration is supplemented only with synthetic thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and choline but no liver extracts a relatively normal rate of growth results and the animals may be carried on such a ration for many months in perfectly normal condition (28, 6, 29). However, if any one of these six synthetic vitamins is omitted, a fatal deficiency in young puppies will occur in 4 to 6 weeks. The use of such a ration for the study of factors involved in blood regeneration was desirable because (a) it would enable us to produce a ration relatively free of any single dietary factor and (b) it would enable us to rule out the effect of possible unknown dietary factors present in natural food materials such as milk.

Since the dogs receiving the synthetic ration supplemented only with synthetic vitamins appeared to grow and develop normally, it would seem that no other dietary factor is necessary. The normal blood stream which the animals maintain on such a ration argues against the necessity of any other factor essential for blood formation, as was concluded from the studies on the milk ration. If such a factor were existent, it was thought probable that the strain of phlebotomy would clearly establish its deficiency.

Accordingly, four older growing dogs were placed on the basal ration used in all of these studies. It has the following composition: sucrose 66 per cent, acid-washed casein 19, cottonseed oil 8, cod liver oil 3, and salt mixture 4.² In addition the dogs received 100 γ each of thiamine chloride and riboflavin, 2 mg. of nicotinic acid, 60 γ of pyridoxine hydrochloride, 500 γ of calcium pantothenate, and 50 mg. of choline chloride per kilo of body weight per day. These vitamins in aqueous solution were given orally twice weekly by pipette. The dogs were bled at varying intervals, usually every 10 to 13 days, over a period of about 7 months. From 15 to 25 per cent of the total blood in the dog was removed at each bleeding. This volume was measured and its hemoglobin determined. At the end

² The salt mixture of Phillips and Hart (30) with an additional 1.2 gm. of $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ per kilo of salt mixture. The mixture contains adequate iron, copper, and manganese but no cobalt salts.

of the bleeding period the dogs were allowed 2 weeks to recover and at that time a blood sample for analysis was taken in the usual manner. The total blood volume and hemoglobin removed over the period, the weight changes, and the blood chemistry and cytology of the four dogs are given in Table II.

Dogs 190 and 191 after 65 and 79 days respectively on this regimen were then supplemented with 20 mg. per day of cobalt as cobaltous chloride. After 82 days the level of cobalt was increased to 40 mg. per day and continued at this level for the final 58 days of the experiment. From Table II it can be seen that these dogs had very little difficulty in maintaining a normal blood stream even when forced to regenerate four to five such blood streams over the experimental period. The cobalt seemed to produce very little if any deleterious effect on blood building, although at the higher

TABLE II

Blood Regeneration Studies on Four Older Growing Dogs Receiving the Synthetic Ration

Dog No. . .	186	188	190	191
Total bleeding period, days	216	216	205	219
Initial weight, kg.	11.8	11.05	15.8	14.2
Final weight, kg. . . .	16.95	12.65	16.9	11.8
Initial blood volume, liters	0 94	0 88	1.26	1.14
Final blood volume, liters	1.36	1 01	1.35	0.95
Blood removed over period, liters	4 82	3 80	5.93	5.09
Hemoglobin removed over period, gm.	515	404	685	636
Final hemoglobin (2 wks. after final bleeding), gm. % .	13 3	12.9	12.2	14.5
Final hematocrit, %	44 0	44.0	40.5	45.0
" mean erythrocyte volume, cu. micra	63	50	66	54
" erythrocyte count, millions per c.mm.	6 97	8 85	6.16	8.39
" plasma iron, γ %	102	52	45	52

levels there was a tendency to anorexia and loss of weight in Dog 191. This was quickly corrected by withdrawal of the cobalt at the end of the experiment. This was not observed in Dog 190 and may be explained on the basis of the greater size of this dog and consequent failure to show the chronic toxicity as quickly. It is of interest that the mean erythrocyte volume of the two dogs receiving cobalt was no lower at the end of the experiment than that of the two dogs without cobalt. Although these values may be considered somewhat subnormal, they are much higher than was observed on the milk ration.

DISCUSSION

The results obtained on the synthetic ration strongly support the thesis that there are no "secondary anemia" curing factors essential in the nutri-

tion of the dog other than those factors supplied. On our particular ration blood regeneration and maintenance were excellent. It is to be emphasized that a completely normal blood stream was produced in 2 weeks after cessation of phlebotomy. Perhaps even less time would be required for this regeneration to take place but this was not studied. Of course, it is possible that by variation of the components of the diet a need might be created for a new factor. But thus far it would seem that of all of the substances necessary for blood building, relatively few are required in the diet and the majority must be synthesized either in the tissues or in the intestinal tract. When, however, the tissues do not receive the few fundamental nutrients in proper quantity, there may be an impairment in the synthesis of the body-made substances and a new type of deficiency could arise.

The studies on the milk ration tend to bear out this conception. Mineralized milk may be regarded as a perfectly complete ration in itself but when used to cure a deficiency already existing or to support an animal under the strain of phlebotomy the border-line concentrations of certain dietary factors in milk may become outright deficient. Synthesis of body-made substances necessary in the regeneration of blood might become impaired. The responses to bile salts and uropterin support this suggestion, since excellent blood regeneration was observed on the synthetic ration in which neither of these substances was included. The factors which might be expected to be deficient in the milk ration under the strain of our experimental conditions are (a) sulfur amino acids, especially cystine and cysteine which are necessary for the detoxication of cobalt, (b) thiamine and nicotinic acid, (c) vitamin D, and (d) essential fatty acids.

The responses to cysteine indicate that a deficiency of this amino acid is clearly possible. Since the animals maintained their weight fairly well in the anemic state, it seems improbable that a significant thiamine or nicotinic acid deficiency could be present. The low content of vitamin D and the essential fatty acids in the milk diet may or may not be important. The optimum calcium-phosphorus ratio in milk would lower the requirement for vitamin D to a minimum but the responses obtained with bile salts might suggest a suboptimal absorption of the vitamin in the anemic animal. The requirement of the dog for linoleic or arachidonic acids for normal growth has not been established. Certainly these fat-soluble substances could not have been furnished by the curative dose of these liver extracts.

The possibility of any amino acid deficiency other than cysteine seems quite remote because of the high quality proteins in milk. Some amino acid deficiencies have been associated with anemias in the various species. Subbarow, Jacobson, and Fiske (31) isolated *l*-tyrosine as the active material in liver extracts which induced reticulocytosis in guinea pigs. They

also found this acid to be a secondary factor in blood formation in the treatment of pernicious anemia (32). Jacobson and Subbarow (33) have made similar claims for tryptophane and list this acid as another accessory factor in the treatment of pernicious anemia. The need for these two amino acids would probably not become apparent on either ration employed by us, since both contained casein in adequate quantity.

Whatever other factors may be involved in addition to those already mentioned, it seems apparent that milk is deficient in a few substances under our experimental conditions which are directly or indirectly involved in blood regeneration. The ultimate deficiency, be it a dietary essential or a substance normally produced by the tissues, can be cured with liver extract which would be expected to contain both types of substances. By adding a number of these pure materials in place of liver extract, good regeneration of blood can be obtained.

The small red blood cells observed in our studies on the milk ration even after prolonged liver therapy recall the observations of Leichsenring and Biester (34) on the blood picture of phlebotomized dogs receiving a highly purified casein-sucrose ration supplemented with 3.2 per cent dried yeast. From their data it seems altogether possible that the cell size reduction observed in their dogs was of the same nature as that observed in our own.

The exact rôle of cobalt in the anemia studies on the milk ration is uncertain. Whether it actually exerts an inhibition of hematopoiesis as suggested by Frost *et al.* (4) cannot be established for certain from these studies. A number of points should be considered in the evaluation of this suggestion.

The hematopoietic response obtained with cysteine which removes cobalt as a biologically inactive complex would indicate that a release from an inhibiting effect of cobalt has occurred. That this release is probably not due to the mere addition of a missing amino acid to an inadequate ration is indicated by the results with the synthetic ration in which excellent regeneration is given with the same protein at a lower percentage intake. The failure of cobalt to inhibit hematopoiesis when added to the synthetic ration indicates that toxic effect on the blood-forming tissues is probably not involved but rather an increased requirement has been created for an essential factor which is adequately met by the synthetic ration and inadequately met by the milk ration. It seems probable that the presence of this nutritionally enormous quantity of cobalt in the ration does have some retarding effect on blood regeneration. However, other evidence indicates that the cobalt is only one of a number of factors involved in the remission failure.

To investigate the rôle of cobalt in the anemia two adult dogs were given the mineralized milk ration without the cobalt supplement. When phle-

botomized they, too, developed a blood picture identical with that observed in the dogs receiving cobalt and showed a similar inability to recover from the anemia. This effect was probably subject to more variation than that exhibited by the dogs receiving cobalt, and a slow remission usually occurred when the bleeding was less severe. Rapid regeneration of blood resulted when these dogs were given liver extracts. It would appear from these studies that an identical deficiency may be produced without added cobalt although the regeneration failure seems much less consistent.

The influence of dietary cobalt on iron transportation and storage in dogs and rabbits has been studied histologically and chemically by Kato (35). Marked increases in total blood iron and liver iron were observed when cobalt was given, but the spleen and bone marrow showed no such deposits unless massive doses of iron were given over a long period of time. The

TABLE III

Influence of Cobalt on Iron Content of Tissues of Dogs Receiving the Mineralized Milk Ration

Dog No.	Daily cobalt supplement	Final hemoglobin concentration	Iron content of tissues per 100 gm. fresh weight	
			Liver	Spleen
	mg.	gm. per cent	mg.	mg.
26	None	14.6	10.8	25.3
27	20	15.7	7.34	23.9
94	20	9.2	8.86	
30	20	8.2	9.52	
23	20	8.4	3.74	
24	20	11.6	4.85	

conclusion was reached that the presence of cobalt increased the utilization of iron.

In our studies on the milk ration the low plasma iron values observed in the anemic dogs suggested an impaired utilization of iron. Iron analyses were therefore performed on liver and spleen samples from some of the dogs used in these studies and are recorded in Table III. The results are somewhat variable but it can be seen that cobalt feeding did not increase the amount of iron in the liver as Kato observed in rabbits. On the other hand, only two of the five dogs fed cobalt showed a low liver iron, the other three being in the normal range. The rôle of cobalt in the storage of liver iron seems doubtful on the basis of these studies although the constant demands on body iron stores brought about by the phlebotomy might have obscured this effect.

The studies on the milk ration bring forth a paradoxical situation in

regard to blood plasma iron content. During the period of negligible hemoglobin formation the plasma iron was very low. However, when liver was supplied and hemoglobin formation was extremely rapid, the plasma iron invariably rose to very high values. This behavior is quite opposite to that observed by Moore *et al.* (36) in their studies on human anemia patients. They found that under conditions of rapid blood regeneration, where iron, available for hemoglobin formation, would conceivably be removed by the hematopoietic tissues, the plasma iron values were low (pernicious anemia patients in remission, and acute hemorrhage patients). Low values were also found in iron deficiency. High values were found after ingestion of large quantities of iron salts, in clinical states characterized by diminished hemoglobin formation (aplastic anemia and pernicious anemia in relapse), and in the hemolytic anemias. The failure on the part of our animals to reflect passively a plasma iron level dependent on hemoglobin synthesis suggests instead that the plasma iron level is *actively* regulated by some substance contained in liver. It is conceivable that an impaired liver function in these deficient animals results in inadequate synthesis of such an iron-mobilizing factor.

SUMMARY

1. Adult dogs rendered anemic by phlebotomy failed to respond to iron and copper when high levels of cobalt were added to the whole milk ration. Rapid regeneration of blood occurred when this ration was further supplemented with whole dry liver or liver extracts.

2. The activity of liver preparations could not be wholly replaced by synthetic B vitamins, bile salts, cysteine, uropterin concentrates, and high levels of iron when fed individually. When these supplements were fed together blood regeneration comparable to that produced by liver preparations was observed. The blood plasma iron, however, was not mobilized as always occurs with liver therapy.

3. Dogs receiving a highly purified ration supplemented only with synthetic vitamins retained their ability to regenerate a normal blood stream after subsection to severe phlebotomy for many months. This regeneration was complete in 2 weeks.

BIBLIOGRAPHY

1. Potter, V. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **126**, 155 (1933).
2. Frost, D. V., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **21**, 93 (1941).
3. Frost, D. V., Potter, V. R., Elvehjem, C. A., and Hart, E. B., *J. Nutrition*, **19**, 207 (1940).
4. Frost, D. V., Spitzer, E. H., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, **134**, 746 (1941).

5. Anderson, H. D., Underwood, E. J., and Elvehjem, C. A., *Am. J. Physiol.*, **130**, 373 (1940).
6. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Nutrition*, **23**, 491 (1942).
7. Evelyn, K. A., *J. Biol. Chem.*, **115**, 63 (1936).
8. Mason, R. D., and Mason, H. L., *Proc. Staff Meetings Mayo Clin.*, **16**, 433 (1941).
9. Gobell, O., *Klin. Woch.*, **18**, 1319 (1939).
10. Dollchen, H., *Klin. Woch.*, **19**, 220 (1940).
11. György, P., Robscheit-Robbins, F. S., and Whipple, G. H., *Am. J. Physiol.*, **122**, 154 (1938).
12. Davis, J. E., *Am. J. Physiol.*, **127**, 322 (1939).
13. Barron, A. G., and Barron, E. S. G., *Proc. Soc. Exp. Biol. and Med.*, **35**, 406 (1937).
14. Griffith, W. H., Paveek, P. L., and Mulford, D. J., *J. Nutrition*, **23**, 603 (1942).
15. Seyderhelm, R., and Tammann, H., *Klin. Woch.*, **6**, 1177 (1927).
16. Seyderhelm, R., Tammann, H., and Baumann, W., *Z. ges. exp. Med.*, **66**, 539 (1929).
17. Takasu, M., *Deutsch. Z. Chir.*, **224**, 240 (1930).
18. Day, H. G., and Stein, H. J., *J. Nutrition*, **16**, 525 (1938).
19. Josephs, H. W., *Bull. Johns Hopkins Hosp.*, **65**, 145 (1939).
20. Hawkins, W. B., Robscheit-Robbins, F. S., and Whipple, G. H., *J. Exp. Med.*, **67**, 89 (1938).
21. Smith, P. W., and Crandall, L. A., *Am. J. Physiol.*, **135**, 259 (1942).
22. Tschesche, R., and Wolf, H. J., *Z. physiol. Chem.*, **248**, 34 (1937).
23. Simmons, R. W., and Norris, E. R., *J. Biol. Chem.*, **140**, 679 (1941).
24. Koschara, W., *Z. physiol. Chem.*, **240**, 127 (1936).
25. Koschara, W., and Haug, H., *Z. physiol. Chem.*, **259**, 97 (1939).
26. McKibbin, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, **128**, 102 (1939).
27. McKibbin, J. M., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, **130**, 365 (1940).
28. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **47**, 365 (1941).
29. Schaefer, A. E., McKibbin, J. M., and Elvehjem, C. A., *J. Biol. Chem.*, **143**, 321 (1942).
30. Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).
31. Subbarow, Y., Jacobson, B. M., and Fiske, C. H., *New England J. Med.*, **212**, 663 (1935).
32. Jacobson, B. M., and Subbarow, Y., *J. Clin. Inv.*, **16**, 573 (1937).
33. Jacobson, B. M., and Subbarow, Y., *J. Am. Med. Assn.*, **116**, 367 (1941).
34. Leichsenring, J. M., and Biester, A., *Minnesota Agric. Exp. Stat., Tech. Bull.* **159** (1939).
35. Kato, K., and Iob, V., *Am. J. Clin. Path.*, **10**, 751 (1940).
36. Moore, C. V., Doan, C. A., and Arrowsmith, W. R., *J. Clin. Inv.*, **16**, 627 (1937).

A COMPARISON OF THE DISPOSITION OF INJECTED GLUCOSE IN TWO STRAINS OF RATS*

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The occurrence of a low tolerance to orally or intraperitoneally administered glucose in adult rats of the Connecticut Agricultural Experiment Station (Yale) strain has been demonstrated (1, 2). The "diabetes" is hereditary (3) and its incidence increases with age (1), approximately 75 per cent of the adult males and 50 per cent of the adult females showing the condition. In many animals there occur unpredictable alternations between low and normal types of tolerances to glucose. The condition has been attributed to some dysfunction of the anterior pituitary gland (4).

Any of a number of biochemical derangements of carbohydrate metabolism may be involved in causing the observed diabetic type of tolerance to administered glucose. These might conceivably include an increased rate of absorption of glucose, a decreased renal excretion of glucose, an impairment in the mechanism of glycogenesis, an excessive degree of glycogenolysis, a lessened rate of carbohydrate oxidation, or a decrease in the rate of transformation of carbohydrate to fat.

The purpose of the present investigation was to obtain information on these possible chemical abnormalities of carbohydrate metabolism in the Yale strain of rat by determining the disposition of injected glucose in these and normal control animals.

EXPERIMENTAL

Healthy adult male and female rats of the Connecticut Agricultural Experiment Station (Yale) and Wistar strains weighing from 250 to 400 gm. were used. They were fed the stock colony ration *ad libitum*, except for a period of 3 days prior to glucose administration, when they were fed daily 10 gm. of stock diet per 250 gm. of body weight ± 1 gm. for each 50 gm. deviation in body weight. This procedure was adopted to lessen variations in stored carbohydrate due to abnormal food intake.

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All animals were fasted 16 to 18 hours before experimentation. The fasting blood sugar level was then determined by the Hagedorn and Jensen method (5) as modified by Kramer and Steiner (6) on blood obtained from a tail vein. Anhydrous glucose (Pfanstiehl) was injected intraperitoneally in doses of 350 mg. per 100 gm. of body weight as an 8.75 per cent solution (38°) from a graduated pipette and the animals were placed in metabolism cages. Experiments on uninjected control rats and Wistar and Yale animals were run simultaneously to compensate for possible variations due to environmental conditions.

At the end of a 5 hour period, the blood sugar level was again determined and the animals were anesthetized with nembutal. Bladder urine was collected quantitatively through a mid-line incision and the sugar content of this urine together with that voided during the 5 hour period was determined by the Hanes (7) macromodification of the Hagedorn and Jensen method. The unabsorbed fermentable carbohydrate content of the peritoneal fluid, collected quantitatively through the incision, was determined by the same method. The liver was then quickly removed and, separately, it and the remaining carcass were rapidly frozen in an ether-solid carbon dioxide mixture. The tissues were finely minced in the frozen state and the carbohydrate content was determined. Liver and carcass glycogen were determined in duplicate upon aliquots of these tissues by a slight modification of the Good, Kramer, and Somogyi method (8). The values were expressed in terms of the fermentable carbohydrate resulting from the hydrolysis of the glycogen. "Free" sugar and total acid-hydrolyzable carbohydrate were also determined on duplicate aliquots of the minced liver and carcass, by the Blatherwick (9) and Cori zinc precipitation (10) procedures respectively. Again as in all other analyses the values were corrected for non-fermentable reducing substances. The portion of the injected glucose deposited in the tissue of the experimental animals was taken to be the amount in excess of that present in the uninjected controls. The data permitted the determination of this value by two means; one, calculated as free sugar plus glycogen, was in excellent agreement with the other obtained through the use of total acid-hydrolyzable carbohydrate. The glucose absorbed and retained minus that deposited represented the amount oxidized or transformed to fat or other substances.

No attempt was made to classify the Yale animals into "normal" and "diabetic" as has been previously done because of the alternations between the two types of glucose tolerances observed in many of these animals.

Results

The average results obtained on uninjected and injected rats of the Wistar and Yale strains, together with standard deviations and significance ratios, are given in Table I.

It is evident from these data that there are differences between the uninjected and injected rats of the two strains. The fasting blood sugar level is significantly higher in the uninjected Yale strain rats, as probably are also the free carbohydrate, glycogen, and total acid-hydrolyzable carbohydrate contents of the liver. Smaller differences of questionable significance exist in the values obtained on carcass carbohydrate.

There are also striking differences between values obtained on the glucose-injected rats of the two strains, except in the case of the absorption and renal excretion of glucose which are almost identical, as previously

TABLE I

Disposition of Injected Glucose in Wistar and Yale Rats

The values are expressed as mg. of glucose per 100 gm. of body weight.

Group	Value	Glucose absorbed	Glucose excreted	Blood sugar	Liver			Remaining carcass		
					"Free" sugar	Glycogen	Total carbohydrate	"Free" sugar	Glycogen	Total carbohydrate
				mg. per cent						
Wistar, uninjected (6 rats)	Average			66	2.1	5.4	10.3	19	253	263
	S.D. (\pm)			10.3	0.4	3.8	5.5	6.6	56.6	53.1
	S.r.*			5.81	4.65	3.15	2.98	1.20	0.58	1.27
Yale, uninjected (6 rats)	Average			77	2.8	10.6	17.4	22	239	285
	S.D. (\pm)			4.7	0.4	4.7	6.7	6.5	68.2	34.0
Wistar, injected (9 rats)	Average	277	3.0	115	6.4	45.3	54.9	42	359	389
	S.D. (\pm)	35.6	1.5	25.0	0.9	21.6	19.1	11.4	60.8	48.7
	S.r.	0.0	0.88	5.19	3.89	2.37	1.96	4.37	6.70	2.26
Yale, injected (10 rats)	Average	277	4.0	219	8.5	30.7	43.2	70	234	346
	S.D. (\pm)	28.4	5.3	90.4	2.2	18.0	19.6	24.5	57.0	69.4

* S.r. expresses the significance of the difference between the averages immediately above and below in the same vertical column. These values are calculated by the procedure given by Sherman (11).

reported (12). The terminal blood sugar values are markedly higher in the Yale strain rats, as has been described (1, 2). The free fermentable carbohydrate content of the livers of the Yale rats is significantly greater and the glycogen and total acid-hydrolyzable carbohydrate contents are significantly lower than those of the Wistar animals. Similar but even greater differences exist in the values obtained on the remaining carcass. The free sugar is higher, whereas glycogen and total carbohydrate values are lower in the Yale animals. The difference was even more pronounced in the more severely "diabetic" animals of the group, the differences paralleling, in general, the magnitude of the terminal blood sugar value.

The values obtained on liver glycogen confirm those previously reported

TABLE II

Disposition of Injected Glucose in Wistar and Yale Rats

All values are expressed as mg. of glucose per 100 gm. of body weight.

Group	Value	Total carcass carbohydrate*			Glucose absorbed and retained	Glucose oxidized, etc.
		Injected rats	Uninjected controls	Difference		
Wistar	Average	453	280	173	274	101
	S.D. (\pm)	51.3	60.7	51.3	35.6	84.8
	Significance ratio	6.08	0.23	5.74	0.0	4.16
Yale	Average	343	274	69	273	204
	S.D. (\pm)	65.2	73.1	65.2	28.4	67.6

* Calculated from "free" glucose + glycogen values. The same values may be calculated by using the figures for "Total carbohydrate" from Table I.

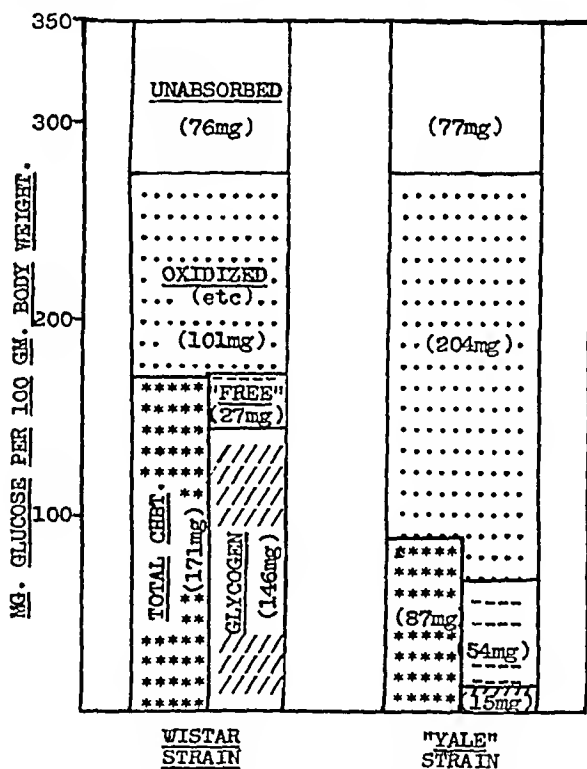


FIG. 1. Disposition of injected glucose in Wistar and Yale strains of rats

(12) on a group of twenty-two Yale rats. The average of the liver glycogen values obtained on these rats following glucose injection was 1.02 per cent, whereas those of ten control Wistar rats averaged 1.92 per cent. Similar

differences were found in the muscle glycogen values. The muscles of the Yale strain of rats averaged 0.67 per cent glycogen, whereas those of the controls averaged 0.76 per cent. The foregoing differences were found to be statistically significant. The significance ratio for the liver glycogen values was 7.5 and for the muscle glycogen, 3.0.

As shown by the data given in Table II, the amounts of glucose oxidized and transformed were much greater in the Yale rats than in the Wistar animals.

A summary of the disposition of the injected glucose in the two strains of rats is given in Fig. 1. The differences between the amounts of "free" sugar and of glycogen present in the tissues of the two strains of injected rats are even more convincing when the values are corrected for the amounts of these substances present in the tissues of uninjected, fasting controls.

DISCUSSION

It is evident from the foregoing data that the outstanding chemical differences between the Yale rats and the Wistar rats involve the amounts of glycogen and "free" sugar in the tissues, and the amount of glucose disposed of by oxidation, by transformation, or by oxidation and transformation.

The lower values for liver and tissue glycogen and the higher levels of free fermentable sugar observed in the Yale rats may be interpreted as evidence of an impairment in the mechanism of glycogenesis in this strain of rats. Such an explanation might satisfactorily explain the entire results obtained in these animals; *e.g.*, the low tolerance to injected glucose, the high blood and tissue sugar levels, and the low glycogen values. The apparently greater amount of carbohydrate oxidized (*etc.*) in these animals might then be regarded as a compensatory glucose-disposing mechanism. An impairment in the ability of the organism to store glycogen in the liver has been suggested by Newburg and Conn (13) as a possible etiological factor in certain types of human diabetes. These authors suggest that the decreased rate of glycogenesis may be related to the presence of excessive amounts of fat in the liver. Comparison of the amounts of fat in the livers of a limited number of normal and "diabetic" rats, however, has revealed no consistent differences between the two strains of animals (14). It has recently been reported (15) that fatty livers produced by feeding low protein diets store as much glycogen as do normal livers.

The present observations may also be satisfactorily explained by postulating that there is an increased rate of glycogenolysis in the Yale strain of rats following glucose injection. Such a hypothesis may be supported by the claim that the Yale strain of rat is more sensitive to epinephrine than

the Wistar animal (4) and also conceivably by the fact that the adrenals of at least male Yale rats are heavier than those of male Wistar rats (16). Further support of the suggestion that an increased rate of glycogenolysis possibly related to hyperadrenal medulla activity may be related to the diabetic tendency in the Yale rat has been obtained from a group of "demedullated" diabetic rats which showed a normal tolerance to glucose following demedullation (14).

The large amount of glucose apparently oxidized by the Yale rat suggests that a lack of insulin is not responsible for the poor tolerance to injected glucose. In this connection, it is interesting that the ratio of glycogen formed to glucose oxidized in the Wistar animals (1.44) is almost exactly the same as that observed by Cori and Cori (17) (value 1.38), whereas the ratio in the Yale rats (0.073) is even less than that obtained by Cori and Cori on insulinized animals (0.87).

SUMMARY

The disposition of intraperitoneally injected glucose was determined in Wistar and Yale strains of rats.

No significant difference was observed in the peritoneal absorption or renal excretion of the glucose in the two strains of animals.

There were, however, significantly smaller amounts of glycogen in the liver and remaining carcass of the Yale rats and significantly larger amounts of "free fermentable" sugar.

From values obtained by difference it appears that the Yale animals dispose of more of the glucose by oxidation, by transformation, or by oxidation and transformation than do Wistar rats.

It is suggested, therefore, that the low tolerance to injected glucose observed in the Yale strain of rats is related to an impairment in the mechanism for the deposition or retention of glycogen in the liver and tissues.

BIBLIOGRAPHY

1. Cole, V. V., and Harned, B. K., *Endocrinology*, **23**, 318 (1938).
2. Orten, J. M., and Devlin, H. B., *Proc. Soc. Exp. Biol. and Med.*, **42**, 632 (1939).
3. Cole, V. V., Harned, B. K., and Keeler, C. E., *Endocrinology*, **28**, 25 (1941).
4. Harned, B. K., and Cole, V. V., *Endocrinology*, **25**, 689 (1939).
5. Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, **135**, 46 (1923).
6. Kramer, H., and Steiner, A., *Biochem. J.*, **25**, 161 (1931).
7. Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).
8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
9. Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., Larson, H. W., and Sawyer, S. D., *J. Biol. Chem.*, **111**, 537 (1935).
10. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **100**, 327 (1933).

11. Sherman, H. C., *Chemistry of food and nutrition*, New York, 5th edition, 602 (1937).
12. Sayers, G., and Orten, J. M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, 140, p. cx (1941).
13. Newburg, L. H., and Conn, J. W., *J. Am. Med. Assn.*, 112, 7 (1939).
14. Orten, J. M., Sayers, G., Sayers, M., and Orten, A. U., *Federation Proc.*, 1, pt. 2, 128 (1942).
15. Treadwell, C. R., King, W. C., Bebb, K. C., and Tidwell, H. C., *J. Biol. Chem.*, 143, 203 (1942).
16. Cole, V. V., and Harned, B. K., *Endocrinology*, 30, 146 (1942).
17. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 70, 557 (1926).

THE EFFECT OF pH ON THE LACTIC ACID FERMENTATION

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In the fermentation of glucose, homofermentative lactic acid bacteria (streptococci and lactobacilli) are generally considered to yield 85 to 98 per cent of the sugar fermented as lactic acid (1-3). Traces of volatile acids, largely acetic, have been reported (2). Some workers have suggested that this may arise from a secondary fermentation or oxidation of lactic acid (4).

In contrast to this, Friedemann (5, 6), in studies of a number of streptococci and pneumococci, has reported the presence of appreciable quantities of formic and acetic acids and ethyl alcohol in the molecular proportion of 2:1:1. Several earlier workers have reported the presence of formic acid among the fermentation products of streptococci (7, 8), some having suggested that the formic acid is formed from lactic acid, especially under alkaline conditions (9). More recently Barron and Jacobs (10) have reported the production of formic and acetic acids from pyruvic acid by resting cells of hemolytic streptococci.

In view of the suggestion (9) that formic acid was formed in an alkaline medium, the present study was undertaken in order to determine whether the pH of the fermentation medium would affect the yields of lactic and volatile acids. A member of the enterococcus group of streptococci was selected for study because of the wide range of pH under which these organisms will grow and ferment. While the results found may not apply to all lactic acid organisms, a partial explanation for the differences found by various investigations is at hand. In addition to the greater yields of volatile acids and ethyl alcohol the formation of a polysaccharide at alkaline reaction, causing the fermentation medium to become viscous, is reported.

Methods

Bacteriological—The culture used, *Streptococcus liquefaciens*, Strain 815, was from the departmental culture collection. It conformed to the cultural and serological characteristics of an enterococcus, Lancefield Group D (11). Resting cell suspensions of this organism have been previously studied and found to produce in excess of 90 per cent of the glucose fermented as lactic acid (12).

The medium used in this study contained 1 per cent tryptone, 0.2 per cent yeast extract, and 0.1 per cent K_2HPO_4 . (In those experiments described in Figs. 1 and 2, 1 per cent K_2HPO_4 was used.) Glucose was sterilized separately and added to the medium aseptically to give a final concentration of 1 per cent. The fermentation flasks were inoculated with 0.1 per cent of a 12 hour culture and incubated at 37°. At the end of the fermentation period sufficient $N H_2SO_4$ was added to bring the pH below 3. In those experiments in which the reaction was held at a constant pH, 2 M Na_2CO_3 was added as required. The indicators, added in aqueous solution, were brom-cresol green for pH 5.0, brom-thymol blue for pH 7.0, thymol blue for pH 9.0, and phenol red for pH $7.5 \pm$.

Chemical—Residual sugar and lactic acid were run on Somogyi filtrates (13), the sugar according to the Folin method (14) and the lactic acid by the method of Friedemann and Graeser (15) or by that of Barker and Summer-son (16), depending on the amount of acid present and the size of the sample available. The two methods for lactic acid gave similar results when run on a given sample. The volatile acids were recovered by steam distillation, an aliquot was titrated, and formic acid determined by the reduction of mercuric chloride to calomel (17). The acetic acid was determined by difference after suitable correction for blanks and recoveries. The identities of the acids present were established by the method of Osburn, Wood, and Werkman (18). The alcohol was identified as ethyl by the same method after oxidation to acetic acid by K_2CrO_7 in acid solution. The alcohol was determined quantitatively by the method of Friedemann and Klaas (19).

Results

Three 500 ml. Erlenmeyer flasks containing 300 ml. of medium were inoculated, adjusted respectively to pH 5, 7, 9, and incubated 24 hours. During the growth and fermentation the pH was held at the starting value. The yield of lactic acid decreased with increased pH (Table I). This was accompanied by an increase in the volatile acids and alcohol in the approximate ratio of two of formic acid to one each of ethyl alcohol and acetic acid, as suggested by Friedemann. In all cases the ethyl alcohol was slightly lower than was expected and the acetic acid correspondingly higher. Since it is known that these organisms oxidize ethyl alcohol to acetic acid (20), it is possible that some oxidation occurred during the fermentation owing to the difficulty involved in keeping anaerobic conditions while alkali was being added. The oxidation-reduction balances would support this contention or indicate that some other reduced product occurs and has not been detected. The low carbon recoveries at more alkaline reactions may be partially accounted for by the formation of a polysaccharide

which accumulated as the fermentation progressed, leaving the medium very viscous. Due to difficulties encountered in the separation and purification of this material, no quantitative estimations were made.

In a series of experiments in which neutralization was accomplished by addition of N NaOH, similar results were obtained but growth was not so good, possibly because overneutralization was difficult to avoid. Similar results were also obtained with a culture of *Streptococcus faecalis*, Strain 10C1.

In a second set of experiments 800 ml. of medium in 1 liter Erlenmeyer flasks were used and 100 ml. samples were taken at intervals as the fermentation progressed in order to determine the rate of formation of the various products. In this case 1 per cent K_2HPO_4 was added to the

TABLE I
Effect of pH on Products

The medium consisted of 1 per cent tryptone, 0.2 per cent yeast extract, 0.1 per cent K_2HPO_4 , 1 per cent glucose. The entire fermentation was carried out at the pH indicated. The products are measured in mM per 100 mM of C_6 (glucose $\times 2$) fermented.

	pH 5.0	pH 7.0	pH 9.0
Lactic acid	87	73	61
Acetic " .	6.1	9.4	15.6
Ethyl alcohol	3.5	7.3	11.2
Formic acid	7.7	16.8	26.4
Carbon recovered, %	95	90	88
Oxidation-reduction balance . . .	1.02	1.18	1.18
Ratio, formic to acetic + ethyl	2:2.7	2:1.96	2:2.03
Glucose $\times 2$ fermented per liter, mM	63.6	112	112

medium instead of 0.1 per cent in order that the conditions would be analogous to those used by Friedemann (5, 6). During the early part of the fermentation period lactic and formic acids increased at approximately equal rates (Fig. 1). At 5 hours the lactic acid accounted for about 40 per cent of the sugar fermented and the volatile products for about an equal quantity. At this time the pH had fallen from an initial value of 7.8 to 6.5. From the 5th hour on (pH 6.5), the curve for lactic acid production is parallel to the curve for sugar fermented, during which time the increase in volatile products is negligible. After the 6th hour the rate of fermentation decreased as the limiting pH for the culture was approached but lactic acid continued to accumulate slowly until it accounted for 71 per cent of the sugar fermented in 24 hours, at which time the fermentation was stopped. These data indicate that the rate of production of volatile acids and alcohol was appreciable only at a pH greater than 6.5.

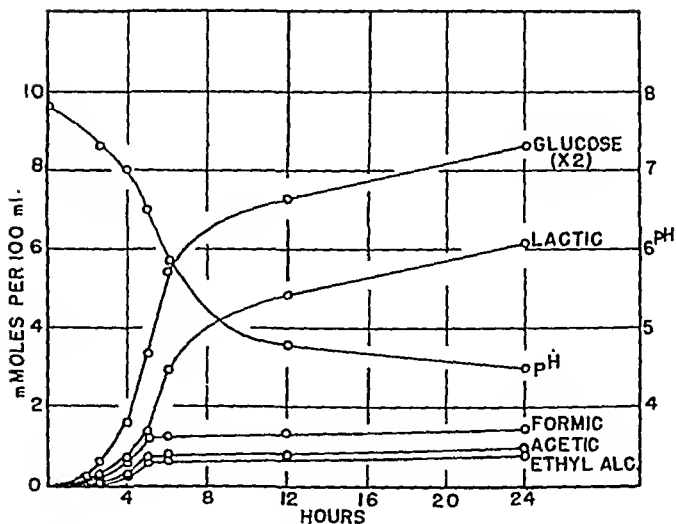


FIG. 1. *Streptococcus liquefaciens*, Strain 815. Amount of glucose used and products formed during fermentation when the pH is allowed to fall. Medium, 0.2 per cent yeast extract; 1 per cent each of tryptone, glucose, and K_2HPO_4 .

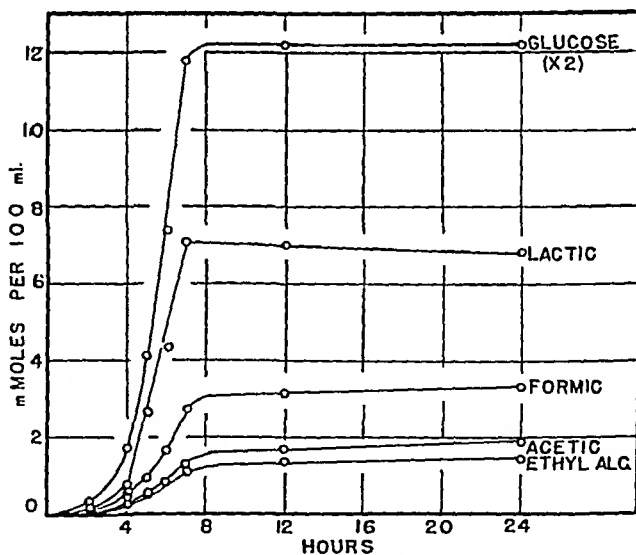


FIG. 2. *Streptococcus liquefaciens*, Strain 815. Amount of glucose used and products formed during fermentation when the pH is held above 7.0. Medium, 0.2 per cent yeast extract; 1 per cent each of tryptone, glucose, and K_2HPO_4 .

A duplicate flask of the medium used in Fig. 1 was inoculated and held at a pH between 7.5 and 8.0 throughout the fermentation period by the

addition of 2 M Na_2CO_3 as required. 100 ml. samples were likewise taken at intervals for analysis. As shown in Fig. 2, all the products continued to accumulate until the sugar was exhausted. In this experiment the volatile acids and alcohol reached about twice the concentration reached in the experiment shown in Fig. 1. At 24 hours the lactic acid accounted for 56 per cent of the sugar fermented, as compared with the 71 per cent in the previous experiment. The carbon recoveries were better than 90 per cent in the early part of the experiment but fell to 82 per cent at 24 hours. Whether this could be completely accounted for by the accumulation of slime was not determined for reasons previously mentioned. In this experiment the analyses indicated a slight decrease in lactic acid from the 7th to the 24th hour.

The slime which forms in alkaline fermentations causes the medium to become very viscous. It can be precipitated by acidification or by the addition of 1 volume of alcohol. Once precipitated, it does not dissolve readily except in normal alkali, from which it can be reprecipitated with acid or alcohol. Due to the limited solubility, its rotation could not be determined. The material which is slowly hydrolyzed by boiling 1 N H_2SO_4 yields some reducing sugar, but not a test for ketose by the Seliwanoff method. The purification and study of this material are being continued.

DISCUSSION

That the fermentation products of such organisms as *Clostridium acetobutylicum* and *Aerobacter aerogenes* are affected by the reaction of the fermentation mixture is well known. The data here presented indicate a similar alteration in the yield of various products among certain strains of streptococci. The data of Friedemann (5, 6) are interpreted to indicate that the effect is present among a number of other strains which he ran under conditions analogous to those used in this work.

The suggestion is offered that the effect of pH is one of the important factors involved in the difference in results reported by such workers as Friedemann with highly buffered media and Langwell (8) with cultures neutralized daily, and the workers who have run their fermentations under the acid conditions optimum for lactic acid production (1, 3).

It should be noted that the ratio of volatile products, formic and acetic acids, and ethyl alcohol is similar among enteric bacteria lacking hydrogenlyase (*Eberthella typhosa*), some pneumococci (5), and *Streptococcus liquefaciens* grown under alkaline conditions.

It is suggested that this factor may also help to explain the results obtained by Friedemann (21) and attributed by him to the age of the culture.

Although large quantities of polysaccharides have been reported to be formed from sucrose by various streptococci (22), we are not aware of pre-

vious reports of the formation of such substances from glucose by these organisms. The significance of this substance should be investigated further.

SUMMARY

A homofermentative lactic acid organism, *Streptococcus liquefaciens*, has been shown to form large quantities of formic and acetic acids and ethyl alcohol in the ratio of 2:1:1 during the fermentation of glucose in a buffered protein-rich medium. This observation confirms the results which Friedemann obtained with a number of streptococci.

The reaction of the fermentation medium was shown to be an important factor in the production of these substances.

When the reaction is held at or above pH 6.5, the combined yields of formic and acetic acids and ethyl alcohol may account for 25 to 40 per cent of the sugar fermented with the yield of lactic acid falling to 60 per cent or less.

The formation of a polysaccharide under alkaline conditions of fermentation is reported.

BIBLIOGRAPHY

1. Tatum, E. L., and Peterson, W. H., *Ind. and Eng. Chem.*, **27**, 1493 (1935).
2. Hewitt, L. E., *Biochem. J.*, **26**, 208 (1932).
3. Whittier, E. O., and Rogers, L. H., *Ind. and Eng. Chem.*, **23**, 532 (1931).
4. Peterson, W. H., Fred, E. B., and Anderson, J. A., *J. Biol. Chem.*, **53**, 111 (1922).
5. Friedemann, T. E., *J. Bact.*, **35**, 527 (1938).
6. Friedemann, T. E., *J. Biol. Chem.*, **130**, 757 (1939).
7. Foster, L. E., *J. Bact.*, **6**, 211 (1921).
8. Langwell, B., *J. Bact.*, **9**, 79 (1929).
9. Walker, E. W. A., and Ryffel, J. H., *Brit. Med. J.*, **2**, 659 (1903).
10. Barron, E. S. G., and Jacobs, H. R., *J. Bact.*, **36**, 433 (1938).
11. Sherman, J. M., *Bact. Rev.*, **1**, 1 (1937).
12. Smith, P. A., and Sherman, J. M., *J. Bact.*, in press (1942).
13. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
14. Folin, O., and Malmros, H., *J. Biol. Chem.*, **83**, 115 (1929).
15. Friedemann, T. E., and Graeser, J. B., *J. Biol. Chem.*, **100**, 291 (1933).
16. Barker, S. B., and Summerson, W. H., *J. Biol. Chem.*, **138**, 535 (1941).
17. Official and tentative methods of analysis of the Association of Official Agricultural Chemists, Washington, 5th edition, 466 (1940).
18. Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 270 (1936).
19. Friedemann, T. E., and Klaas, R., *J. Biol. Chem.*, **115**, 47 (1936).
20. Greisen, E. C., Thesis, Cornell University (1942).
21. Friedemann, T. E., *J. Bact.*, **43**, 29 (1942).
22. Niven, C. F., Jr., Smiley, K. L., and Sherman, J. M., *J. Biol. Chem.*, **140**, 105 (1941).

THE EFFECT OF SULFAGUANIDINE ON RAT GROWTH AND PLASMA PROTHROMBIN*

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Marshall and coworkers (1) first prepared the drug sulfaguanidine and described it as an effective bacteriostatic agent for intestinal organisms. It appeared to us, therefore, to be a useful tool for the inhibition of synthesis of nutritional factors by intestinal bacteria. We found (2) that when it was included in a synthetic ration at a 0.5 per cent level the drug greatly reduced the growth of young rats, and that liver extract and *p*-aminobenzoic acid antagonized this effect. These results have been confirmed by Mackenzie *et al.* (3). Though vitamin K was known to be supplied to the rat by its intestinal flora, we found no lengthening of the clotting time of whole blood from rats receiving sulfaguanidine (2). Because the prothrombin level can be considerably reduced before a lengthening of the clotting time of whole blood or plasma is detected (4, 5), we have extended this work to include determinations of the plasma prothrombin time of diluted plasma from rats receiving the drug. The results show that the retarded growth caused by sulfaguanidine is accompanied by a state of hypoprothrombinemia, as measured by the prothrombin time of 12.5 per cent plasma. These effects of sulfaguanidine can be counteracted by several substances.

EXPERIMENTAL

The basal ration consisted of sucrose 76, purified casein 18, salts 4,¹ corn oil 2, choline hydrochloride 200 mg., nicotinic acid 2.5 mg., calcium pantothenate 2 mg., and 0.3 mg. each of thiamine, pyridoxine, and ribo-

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for supplies of thiamine, nicotinic acid, vitamin B₆, calcium pantothenate; to the Abbott Laboratories, North Chicago, Illinois, for haliver oil; and to the Calco Chemical Division, American Cyanamid Company, Bound Brook, New Jersey, for sulfaguanidine.

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¹Salts 4 (Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 138, 459 (1941)).

flavin. 2 drops of haliver oil were given weekly to each rat. Weanling male rats were used in all experiments and the growth rate was determined over a 5 week period. The prothrombin time was determined by the modified method of Quick on plasma diluted with saline to a concentration of 12.5 per cent (4, 6). Blood was obtained by heart puncture from animals under ether anesthesia. The thromboplastin used in the determination was prepared from rabbit brain. Plasma from rats is relatively uniform in its prothrombin activity; the average clotting time of 12.5 per cent plasma is 39 seconds (range 36 to 45 seconds) (6). It was found that inclusion of 0.5 per cent sulfaguanidine in the ration increased the prothrombin time to a maximum value in 4 weeks, and subsequent determinations showed no significant increase or decrease between the 4th and 8th weeks. The values given in Tables I to III are averages of several determinations on each rat made between the 4th and 8th weeks on the ration. To determine the chemical stability and solubility of the sulfaguanidine-antagonizing factor of liver extract, several preparations from this material were made.

Liver Preparations

Heat Stability—A neutral 20 per cent solution of liver extract and a 20 per cent solution of liver extract containing 50 gm. of concentrated H_2SO_4 per liter were autoclaved for 5 hours at 120° (Experiments 4 and 5, Table I). A 20 per cent solution of liver extract containing 45 gm. of solid NaOH per liter was heated on the steam bath for 5 hours at 87° (Experiment 6). The fractions were neutralized and concentrated in the usual manner for feeding as daily supplements.

Norit Fractions—A 4 per cent solution of liver extract acidified with H_2SO_4 to thymol blue was stirred twice for periods of 1 hour with an amount of norit equal to one-half the weight of liver extract in solution. After filtering and washing, the adsorbed material was eluted from the norit by stirring with three successive portions of 5 per cent NH_4OH , each portion being equivalent to 10 times the weight of the norit. Each fraction was neutralized and concentrated for feeding as a daily supplement (Experiments 7 and 8, Table I).

Ether Extractions—One portion of the norit eluate was acidified to thymol blue and one alkalized to phenolphthalein and each extracted continuously for 3 days with purified ethyl ether. The ether was removed *in vacuo* and the fractions prepared for feeding as above (Experiments 9 to 12, Table I).

Calcium Precipitation—A portion of norit eluate equivalent to 128 gm. of liver extract was concentrated to 25 ml. 10 gm. of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were added, and the solution neutralized with 10 per cent KOH and added to

300 ml. of 95 per cent alcohol. The precipitate was washed twice with 95 per cent alcohol and the calcium removed from its aqueous suspension with Na_2SO_4 (Experiments 13 and 14).

Lead Precipitation—A lead precipitation was performed on the norit eluate with lead subacetate by the usual procedure and the lead removed from both fractions with H_2S . The PbS from each fraction was washed

TABLE I

Growth Rate and Prothrombin Time of Rats Receiving Synthetic Ration with 0.5 Per Cent Sulfaguanidine Plus Preparations of Liver Extract and Grass Juice*

Preparations fed, equivalent to 0.6 gm. of liver extract.

Ex- periment No.	Daily supplement	No. of rats	Weekly growth	Average pro- thrombin time
			cm.	sec.
1	Control, no sulfaguanidine	6	24.8	38
2	" with sulfaguanidine	28	8.7	113
3	0.3 gm. liver extract	7	32.1	38
4	Neutral, autoclaved liver extract	3	32.9	
5	Acid, autoclaved liver extract	3	14.3	
6	Alkali-treated " "	3	27.6	
7	Norit filtrate	8	17.1	
8	" eluate	8	28.2	
9	Acid ether extract	3	14.4	
10	" " residue	3	22.1	
11	Alkaline ether extract	3	16.1	64
12	" " residue	3	23.3	46
13	Calcium filtrate	3	13.0	
14	" ppt.	3	25.4	
15	Lead ppt. + lead filtrate	3	11.5	
16	Superfiltrol eluate	3	22.1	45
17	Fullers' earth filtrate + butanol eluate	3	12.0	
18	0.6 gm. grass juice†	3	29.6	35

* Prothrombin time of 12.5 per cent plasma (1 part plasma, 7 parts saline).

† Grass juice supplied by the Cerophyl Laboratories, Inc., Kansas City, Missouri.

with hot water and the washings added to the corresponding fraction (Experiment 15, Table I).

Fullers' Earth Adsorption—A solution of norit eluate acidified to thymol blue with H_2SO_4 was shaken on a mechanical shaker with two portions of fullers' earth, each equivalent in weight to the solid material in solution, for periods of 1 hour. After the fullers' earth had been filtered and washed, an elution of the adsorbed material was attempted by refluxing twice with *n*-butanol. The butanol was removed *in vacuo* and the fractions prepared for feeding (Experiment 17, Table I).

Superfiltrol Eluate—This was prepared by the procedure of Hutchings *et al.* (7) (Experiment 16, Table I).

DISCUSSION

The addition of sulfaguanidine to the synthetic diet reduced the growth of the rats from the normal rate of 25 gm. per week to about 9 gm. The average prothrombin time of the 12.5 per cent plasma from these animals was prolonged from the normal value of 36 to 45 seconds (6) to an average of 113 seconds. Liver extract (Table I) or *p*-aminobenzoic acid (Table II) counteracts both effects of the sulfaguanidine, while vitamin K (2-methyl-1,4-naphthohydroquinone diacetate) prevents only the hypoprothrombinemia.

TABLE II

Growth Rate and Prothrombin Time of Rats Receiving Synthetic Ration with 0.5 Per Cent Sulfaguanidine, Plus p-Aminobenzoic Acid Orally and Subcutaneously, and Vitamin K (2-Methyl-1,4-naphthohydroquinone Diacetate) Orally*

Ex- periment No.	Daily supplement	No. of rats	Weekly growth	Average pro- thrombin time
			gm.	sec.
1	3.0 mg. <i>p</i> -aminobenzoic acid	12	22.4	47
2	300 γ " "	6	22.4	43
3	150 γ " "	6	20.3	52
4	50 γ " "	6	14.1	105
5	300 γ " " subcutaneously	8	20.4	53
6	150 γ " " "	8	20.1	48
7	1 mg. vitamin K per kilo ration	10	9.3	40

* Prothrombin time of 12.5 per cent plasma.

With the exception of vitamin K all preparations that prevent the sulfaguanidine from inducing a hypoprothrombinemia also antagonize the growth effect, and preparations which do not counteract the hypoprothrombinemia produce poor growth. The factor in liver extract responsible for the effect on the prothrombin level (or activity) as reflected by the clotting time of 12.5 per cent plasma is apparently not vitamin K, for it remains in the alkaline residue after prolonged extraction with ether. Because the two effects produced by liver extract preparations are parallel in all cases thus far determined, they can for the present be considered due to a single factor. This liver factor appears to be distinct also from *p*-aminobenzoic acid, for the following reasons: (1) A bacteriological assay (8) made by Mr. J. O. Lampen on several active preparations showed less

than 1 γ of *p*-aminobenzoic acid present per rat day dose, whereas a daily dose of 150 γ of the crystalline compound is required to produce both the growth and the prothrombin effects. (2) The factor, unlike *p*-aminobenzoic acid, is relatively unstable to heat in an acid solution. (3) Continuous ether extraction does not remove it from an acid solution. (4) It appears in the calcium-EtOH precipitate rather than in the filtrate.

It is of interest that the several properties determined for the sulfaguanidine-antagonizing principle of liver correspond very closely with a norit eluate factor required for the growth of *Lactobacillus casei*. This factor has been described by Hutchings *et al.* (7) and has been designated as "folic acid" by Williams and coworkers (9). It has also been shown to be essential in the diet of the chick (10). The properties which correspond are the following: a greater stability in alkaline solution than in acid, insolubility in organic solvents such as ether and *n*-butanol, precipitation by calcium chloride, destruction during lead fractionation by removal of lead with H_2S , and adsorption by fullers' earth and superfiltrol. Both factors are abundant in grass juice.

The hypothesis of intestinal synthesis of nutritional factors is a convenient basis for explaining the sulfaguanidine effects. According to this explanation the drug inhibits synthesis of essential factors by the intestinal flora. Any substance which antagonizes the drug action does so either by supplying the factors normally produced by the intestinal organisms, or by counteracting the effect of the drug on the bacteria. This hypothesis seems especially plausible, since vitamin K has been shown by other means to be synthesized in the intestine of the rat (11). However, the sulfaguanidine-antagonizing action of *p*-aminobenzoic acid administered parenterally (Table II) suggests that the effects cannot be explained on the basis of changes in intestinal flora alone, but may be due to a toxic action of the sulfaguanidine on certain tissues of the rat, which is counteracted by *p*-aminobenzoic acid. Thus the exact mode of action of the drug is still obscure. Other effects from the prolonged administration of sulfaguanidine to rats are described by Mackenzie *et al.* (3) and Daft and coworkers (12).

During the course of the work with sulfaguanidine Dr. A. D. Welch of Sharp and Dohme, Inc., Glenolden, kindly supplied us with the new drug, sulfasuxidine (succinylsulfathiazole). We have found that it also reduces the growth rate and causes changes in the prothrombin level (or activity) similar to those observed with sulfaguanidine, and these effects are antagonized in a similar manner by vitamin K and liver extract (Table III). Since this work was completed, Welch has reported (13) the same growth results with sulfasuxidine, but states that, unlike sulfaguanidine, sulfasuxidine is not antagonized in the rat by *p*-aminobenzoic acid. This suggests that the mode of action of the two drugs may not be alike.

To our knowledge there is no report in the literature suggesting that sulfonamide drugs, clinically or experimentally, interfere with the blood clotting mechanism by altering the level (or activity) of prothrombin. However, there are many clinical reports (14) of liver injury caused by these compounds. Since prothrombin is presumed to be formed in the liver, it is possible that the plasma of patients suffering from liver injury due to sulfonamide therapy may indicate prolonged prothrombin times, especially if the prothrombin time is measured on diluted plasmas. It appears to us that this possibility merits consideration by clinical investigators, and that vitamin K, liver extract, or some other substance may be used to prevent a possible hypoprothrombinemia from arising in conjunction with sulfonamide therapy.

TABLE III

Growth Rate and Prothrombin Time of Rats Receiving Synthetic Ration with 0.5 Per Cent Sulfasuzidine Plus Liver Extract and Vitamin K (2-Methyl-1,4-naphtho-hydroquinone Diacetate)*

Experiment No.	Daily supplement	No. of rats	Weekly growth	Average prothrombin time
			gm.	sec.
1	Control	9	11.5	85
2	0.3 gm. liver extract	6	30.1	42
3	10 mg. vitamin K per kilo ration	3	8.2	44

* Prothrombin time of 12.5 per cent plasma.

SUMMARY

1. Sulfaguanidine reduces the growth rate of young rats on a synthetic ration, and this effect is accompanied by a state of hypoprothrombinemia as measured by the prothrombin time of 12.5 per cent plasma.

2. *p*-Aminobenzoic acid and a distinct factor in liver extract counteract both effects of sulfaguanidine. Vitamin K counteracts the prothrombin effect only.

3. The mechanism of action of sulfaguanidine and its antagonists in the rat is discussed.

4. The possible bearing of these findings on sulfonamide therapy is indicated.

BIBLIOGRAPHY

1. Marshall, E. K., Jr., Bratton, A. C., White, H. J., and Litehfield, J. T., Jr., *Bull. Johns Hopkins Hosp.*, 67, 163 (1940).
2. Black, S., McKibbin, J. M., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 47, 308 (1941).

3. Mackenzie, J. B., Mackenzie, C. G., and McCollum, E. V., *Science*, **94**, 518 (1941).
4. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941).
5. Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **142**, 941 (1942).
6. Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, **23**, 589 (1942).
7. Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).
8. Lampen, J. O., and Peterson, W. H., *J. Am. Chem. Soc.*, **63**, 2283 (1941).
9. Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, **63**, 2284 (1941).
10. Mills, R. C., Briggs, G. M., Jr., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, **49**, 186 (1942).
11. Greaves, J. D., *Am. J. Physiol.*, **125**, 423 (1939).
12. Daft, F. S., Ashburn, L. L., Spicer, S. S., and Sebrell, W. H., *Pub. Health Rep., U. S. P. H. S.*, **57**, 217 (1942).
13. Welch, A. D., *Federation Proc.*, **1**, pt. 2, 171 (1942).
14. Long, P. H., and Bliss, E. A., *The clinical and experimental use of sulfanilamide, sulfapyridine, and allied compounds*, New York, 280-282 (1939).

THE BIOCHEMICAL DEFECT IN NICOTINIC ACID DEFICIENCY

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The discovery by Elvehjem *et al.* (1) that nicotinic acid was the pellagra-preventive factor of Goldberger (2) came shortly after nicotinamide had been shown to be a component of codehydrogenases I and II (3, 4). Since then it has been assumed that the manifestations of nicotinic acid deficiency, including death, in human pellagra and in canine blacktongue are the results of the failure of those tissue respiratory mechanisms which involve the pyridine nucleotides (5). However, *in vitro* comparison of the tissues of normal dogs and dogs in blacktongue has shown no significant difference in their ability to oxidize glucose or lactic acid (6). After having performed simultaneous nicotinic acid and coenzyme analyses on the tissues of normal dogs and dogs in blacktongue, we were forced to conclude that death in blacktongue is not due to a simple deficiency of cozymase and the consequent failure of tissue respiration in the tissues examined (7), but must be the result of some other combination of circumstances.

EXPERIMENTAL

The first signs of blacktongue usually appear within 2 months after dogs are placed on our diet (6, 7) and the disease generally becomes acute within the following 2 weeks. During the period preceding blacktongue, dogs lose about 10 per cent of their body weight. At the onset of acute blacktongue, when the mouth lesions are severe, dogs frequently lose 20 to 30 per cent of their original body weight within 5 days. These phenomena are illustrated by the history of Dog 11 in Fig. 1. Such a rapid decline can be attributed only to the loss of fluids. Following this period the animals are always anuric and refuse to eat or drink. There is no preliminary period of diuresis but the fluid loss is frequently aggravated by a severe, bloody diarrhea. It seemed possible, therefore, that dehydration and electrolyte imbalance might be factors of primary importance in death due to nicotinic acid deficiency.

To test this hypothesis a series of dogs in blacktongue was given large

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volumes of 0.9 per cent NaCl solution subcutaneously twice daily. The amount given was somewhat less than 10 per cent of the body weight per day. Therapy was instituted sometimes when the first red patches appeared in the dogs' mouths, sometimes when acute blacktongue became apparent, and at others when death seemed certain within 24 hours. In twenty-nine of the 57 dogs studied, within about 2 weeks the signs of blacktongue dramatically disappeared. The mouth lesions healed, the dogs' appetites returned to normal, and their weights returned towards what they had been at the onset of blacktongue. The history of a typical dog is diagrammatically presented in Fig. 2.

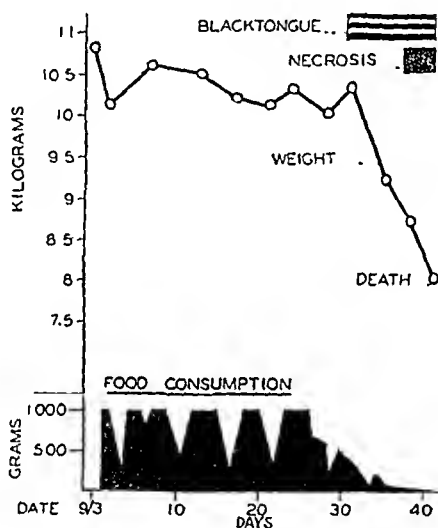


FIG. 1. History of a typical, untreated dog (No. 11) on a blacktongue-producing diet. The blocks represent the incidence and duration of the appearance of any of the clinical signs of blacktongue and of the acute symptoms of blacktongue (necrosis) respectively.

As soon as the dogs resumed eating, the saline therapy was stopped. Almost all dogs were maintained alive for at least 30 days after blacktongue had subsided, while some remained alive with no further administration of fluids for 180 days. The mean survival period of twenty-nine dogs was 75 days. At the end of this period some of the dogs again presented the classical picture of blacktongue, but twelve dogs died with no obvious sign of blacktongue. They became progressively weaker and were unable to stand for the last few days. The characteristic lesions and inflammation of the oral mucosa were completely absent. The breathing of these animals became progressively more shallow and death ensued with no convulsive activity.

Several dogs presented an unusual picture before they died. There appeared first stiffening of the neck muscles, followed by a complete rigidity of the legs which remained for 3 to 7 days before death. At autopsy, even dogs which had been maintained alive for 4 months after the passing of acute blacktongue showed no striking changes from normal other than the disappearance of almost all depot fat. Quite frequently death seemed to be due to pneumonia and almost invariably the heart was dilated. More complete, systematic pathological studies are now in progress.

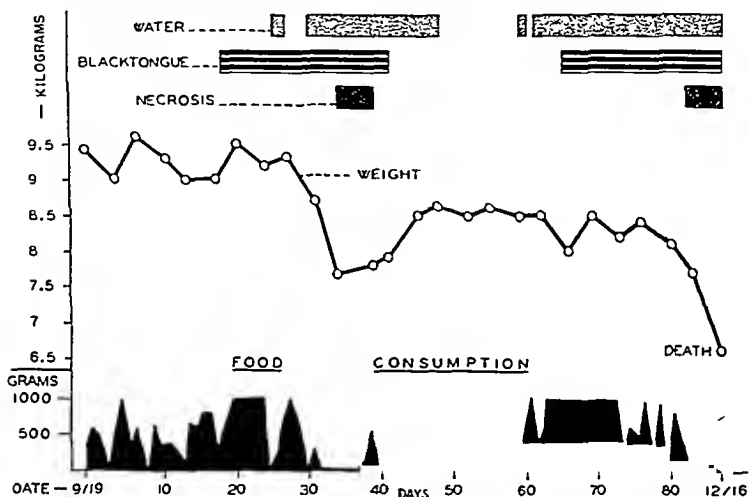


FIG. 2. History of a typical dog (No. 22) on a blacktongue-producing diet treated with physiological saline solution. The blocks represent the period in which fluids were administered, the incidence and duration of any of the clinical signs of blacktongue, and of the acute symptoms of blacktongue (necrosis of the oral mucosa), respectively.

Not all dogs responded as well as the group represented by Dog 22 in Fig. 2. Although the necrotic lesions of nineteen dogs healed, a patchy redness persisted and their appetites were only partially improved. Nevertheless, a number of such animals have lived on for as long as 120 days. These dogs lost weight steadily throughout this period. Only nine dogs completely failed to respond to the administration of the saline solution and died in the usual fashion. Examinations were made of the blood, urine, and tissues of normal dogs and dogs in acute blacktongue as well as those protected by the saline therapy.

Blood Picture in Nicotinic Acid Deficiency—In Tables I and II is presented a summary of the blood picture in canine nicotinic acid deficiency.

Samples were taken from dogs in acute blacktongue just before the first administration of saline. The dehydration and hemoconcentration of blacktongue are shown by the rise in hemoglobin, hematocrit, and plasma proteins. The non-protein nitrogen was markedly elevated; in one dog whose life was subsequently prolonged by saline it rose to 145 mg. per cent. The blood glucose, chloride, and CO₂-combining power were all low.

TABLE I
Blood Picture in Nicotinic Acid Deficiency

The methods used for these determinations as taken from the literature were for non-protein nitrogen (8), glucose (9), CO₂-combining power (10), plasma proteins by Kjeldahl, total base and chlorides by electroanalysis (11), lactic acid (12), and pyruvic and other α -keto acids (13). The figures in parentheses represent the number of dogs in each group.

Condition	Glucose gm. per cent	Non-protein nitrogen mg. per cent	Hemoglobin gm. per cent	Hematocrit vol. per cent	CO ₂ -combining power m.eq. per l.	Chloride m.eq. per l.	Total base m.eq. per l.	Lactic acid mg. per l.	Pyruvic acid mg. per cent	Other α -keto acids mg. per cent
Normal (9)	5.9	0.67	30	83	56	100	166	3.8	0.8	0.
Blacktongue (7)	6.9	0.42	84	58	33	91	163	4.6	1.9	0.
Cured by saline (11)	5.6	0.44	21	80	46	98	168	4.3	1.3	0.
In extremis (11)	5.1	0.35	56	70	33	99	165	6.1	1.1	0.

TABLE II
Blood Picture in Nicotinic Acid Deficiency

The figures in parentheses represent the number of dogs in each group.

Condition	Hematocrit per cent	Hemoglobin gm. per cent	Red cell count millions per c.mm.	White cell count thousands per c.mm.	Sedimentation rate mm. per hr.
Normal (9)	50.0	16.0	6.9	13.8	8
Blacktongue (7)	54.0	16.6	7.4	5.1	18
Cured by saline (11)	32.0	8.8	5.9	5.0	
In extremis (11)	20.0	5.9	2.6	2.5	65

The moderately severe acidosis, in the presence of a low chloride level, could not be accounted for by the observed increases in pyruvic and lactic acids and must have been due to some other organic acids. The white cell count was greatly decreased.

The samples from dogs "cured by saline" were taken 2 weeks after the disappearance of blacktongue and showed a return to normal in the plasma proteins, non-protein nitrogen, glucose, and chloride. The CO₂-combining

power moved toward normal in this period and the white cell count remained unchanged, but severe decreases occurred in the hemoglobin, hematocrit, and red cell count.

The samples from dogs *in extremis* were taken when it was judged that death was inevitable within 48 hours, just before they were sacrificed for tissue analysis. The data show that during the maintenance period, after recovery from blacktongue, the plasma proteins decreased a little and the non-protein nitrogen rose, but not as much as in blacktongue. The glucose decreased, again less than in blacktongue, and the CO_2 -combining power fell to the level in blacktongue. Blood chloride remained normal, while lactic and pyruvic acids were somewhat elevated. The most prominent change during this maintenance period was the development of a profound anemia in which no immature red cells were to be seen. The decreased white cell count at all stages of deficiency was not accompanied by any change in the differential count. A more complete study of this anemia is now in progress.

Urinary Constituents in Nicotinic Acid Deficiency—The urine of a group of dogs was examined at the start of the experiment, in blacktongue for the 24 hours following the first administration of saline solution, 30 days later, and for the 24 hour period preceding death *in extremis*. The data are summarized in Table III. After blacktongue almost all dogs were in negative nitrogen balance. They ingested approximately 2.0 gm. of nitrogen and excreted 2.8 gm. daily. During blacktongue, there was always a large rise in the excretion of creatine; this fell off when the dogs had returned to an apparent normality, but never returned to the basal level determined for the dogs when first put on the blacktongue-producing diet. In acute blacktongue rather large quantities of a poorly characterized protein appeared in the urine. The material was not always found by the usual heat coagulation test, even after cooling and acidification. This seemed to be determined by the concentration in which it occurred. However, in the urine of all dogs in blacktongue there did appear a protein-like substance which was precipitated by the addition of 2 volumes of alcohol or half saturation with ammonium sulfate. This material, when redissolved, gave a positive biuret test. The amount excreted by dogs cured by saline therapy varied considerably from day to day and was completely absent in one-third of the urine samples examined. It invariably reappeared in large quantities in dogs *in extremis*.

Nicotinic Acid and Coenzyme Content of Dog Tissues in Nicotinic Acid Deficiency—When it seemed likely that a given dog could not survive more than another 48 hours, the animal was sacrificed by decapitation and tissue samples were taken for analysis. Nicotinic acid determinations were performed by the procedure of Dann and Handler (16) and the pyridine nu-

cleotides were estimated by the V factor technique of Kohn (17, 18). These data are summarized in Table IV. The values for normal dogs and those in blacktongue have been presented elsewhere (7) and are included here for comparison. Mean values are given for twenty-one dogs which were protected by saline for at least 30 days. The average period of survival was 75 days. Values are also given for a subgroup comprising those dogs which survived more than 90 days. For these, the average survival period was 115 days. Pyridine nucleotides are expressed as micrograms of diphosphopyridine nucleotide per gm. of tissue (wet weight). The validity of this mode of expression has already been discussed (18). By bound nicotinic acid is meant that nicotinic acid which was found as pyridine nucleotides; this figure is obtained by dividing the figure for cozymase by 5.4. Un-

TABLE III

Urinary Constituents in Nicotinic Acid Deficiency

The methods used for these determinations were as follows: nitrogen by Kjeldahl, creatinine and creatine by a modification of the procedure of Folin and Wu (14), phosphorus (15), pH on the glass electrode, and ammonia by formol titration. The figures in parentheses represent the number of dogs in each group.

Condition	Sugar	Protein	Acetone bodies	Volume	Nitrogen			Creatinine		Creatine		Phosphorus		pH	Ammonia
					cc. per day	gm. per day	mg. per cc.	mg. per day	γ per cc.	mg. per day	γ per cc.	mg. per day	mg. per cc.		
Normal (9)	—	—	—	370	3.0	8.1	20.1	60	1.6	4.3	445	1.2	6.8	22	
Blacktongue (20)	—	+	—	340	3.7	10.4	20.1	62	12.7	37.1	380	1.1	6.6	30	
Cured by saline (8)	—	+	—	310	2.8	8.3	20.6	61	2.7	8.0	340	1.1	7.1	25	
<i>In extremis</i> (7)	—	+	—	270	2.0	7.3	11.1	41	1.9	7.1	200	0.8	5.7	17	

bound nicotinic acid is the arithmetical difference between total and bound nicotinic acid.

There was a progressive decrease in the nicotinic acid content of liver and muscle in deficiency. In liver this decrease occurred largely in the bound fraction until the blacktongue disappeared. Beyond this point the bound and unbound fractions decreased at about the same rate. In dogs *in extremis* the pyridine nucleotides represented only one-fifth, in blacktongue about one-third of the total nicotinic acid present, compared with more than half in the normal dog. In muscle, while the total nicotinic acid content decreased in dogs *in extremis* to 60 per cent of the normal value, this drop was confined to the bound fraction and the unbound fraction actually doubled. The values obtained for the pyridine nucleotide content of liver and muscle of dogs *in extremis* were astonishingly low and certainly must be

considered as a possible cause of death in these animals; but despite the severe decrease in liver and muscle cozymase there was only a relatively small increase in the blood lactic acid concentration of these animals. As in the rat (16), the nicotinic acid of dog kidney cortex appeared to exist en-

TABLE IV

Nicotinic Acid and Coenzyme Content of Tissues in Canine Nicotinic Acid Deficiency

All values are expressed as micrograms per gm. of tissue (wet weight). The figures in parentheses represent the number of dogs in each group.

Tissue	Condition	Nicotinic acid					Diphosphopyridine nucleotide	
		Total		Bound		Unbound	Range	Mean and S.E.
		Range	Mean and S.E.	Range	Mean and S.E.			
Liver	Normal (10)	90-233	153 \pm 12	53-133	81 \pm 8.5	72	290-720	438 \pm 46
	Blacktongue (10)	84-117	101 \pm 4.3	21- 57	34 \pm 3.5	67	114-309	184 \pm 19
	Protected 30 days (21)	43-166	73 \pm 6.3	4- 70	26 \pm 4.6	47	20-378	140 \pm 25
	Protected 90 days (11)	43- 86	58 \pm 4.0	4- 22	11 \pm 1.4	47	21-120	64 \pm 7.5
Muscle	Normal (10)	59- 80	71 \pm 1.9	47- 66	57 \pm 2.2	14	250-356	310 \pm 12
	Blacktongue (10)	32- 81	59 \pm 5.3	16- 60	41 \pm 5.2	18	86-360	222 \pm 28
	Protected 30 days (21)	21- 82	50 \pm 4.1	1- 67	25 \pm 3.7	24	7-344	139 \pm 20
	Protected 90 days (11)	21- 69	44 \pm 6.0	1- 38	16 \pm 2.3	28	7-204	89 \pm 12
Kidney cortex	Normal (10)	75-117	95 \pm 3.4	72-106	93 \pm 3.3	?	388-570	504 \pm 18
	Blacktongue (10)	75-104	96 \pm 2.7	75-105	92 \pm 2.8	?	416-574	500 \pm 15
	Protected 30 days (21)	37-106	67 \pm 3.9	27- 85	63 \pm 3.7	?	150-459	340 \pm 20
	Protected 90 days (11)	37- 90	65 \pm 5.4	27- 85	57 \pm 5.0	?	150-459	294 \pm 27

tirely as pyridine nucleotides; this situation was not altered in blacktongue nor did the concentration of these substances decrease. As the deficiency progressed past blacktongue, the total nicotinic acid decreased and there may have appeared very small amounts of unbound nicotinic acid.

To evaluate further the function of the salt solution in effecting these changes, attempts were made to substitute isotonic glucose solution, amino

acid solutions, and salt tablets for the 0.9 per cent NaCl solution. The technique used was identical with that described for the saline solution. Of ten animals which received the isotonic glucose solution, only one showed any beneficial effects. This dog appeared to recover from the typical blacktongue crisis for 1 week, then showed the usual blacktongue symptoms for 2 weeks, and died. Amino acid solutions were supplied as a 2.5 per cent solution of amigen.¹ Of ten animals receiving this solution only two showed any beneficial effects and these, again, were short lived and may, perhaps, have been due to the small concentration of salt in the preparation. Five dogs have been supplied 3 gm. daily of salt tablets. While the response in these animals was not quite as rapid as the response to isotonic salt solution, the effects of the two forms of therapy seemed to be in all ways comparable. Further, the effect did not seem to be the result of thirst induced by the administration of salt, since each dog refused water until the mouth lesions were at least partially healed. At this point their appetites returned and they commenced to eat and drink simultaneously.

DISCUSSION

The findings presented here have served to clarify somewhat the chain of events leading to death in nicotinic acid deficiency. However, at present, it is not possible to state the underlying factor which is responsible for the dehydration and electrolyte imbalance. The fluid loss is not solely due to the bloody diarrhea, since many dogs never do exhibit it. Nor can the dehydration or electrolyte imbalance be ascribed simply to the prolonged period of anorexia, since quite frequently dogs have been observed to die within 24 hours of the time when they first refused to eat or drink. The lack of appetite and thirst, however, may be associated simply with the pain occasioned by the severe necrosis of the oral mucosa. On the other hand, the possibility must be borne in mind that these animals do not feel thirsty.

It does seem that a rather close analogy may be drawn between the crisis in blacktongue and that seen in adrenal insufficiency as observed in the experimentally adrenalectomized animal or in Addison's disease in the human. The effect of the administration of salt solutions and of salt itself, the decreased blood chloride and glucose, the renal impairment, all are seen in both conditions and suggest a dysfunction of the adrenal cortex attendant upon a deficiency of nicotinic acid or its physiological derivatives. These findings lend support to the conclusion earlier reached (6, 7) that death in canine blacktongue (and perhaps, in pellagra) is not due to a deficiency of the pyridine nucleotides with consequent failure of tissue respira-

¹ A tryptic digest of casein obtained from Mead Johnson and Company, Evansville, Indiana.

tion, in the organs studied. They do not, however, suggest the exact nature of the relationship between nicotinic acid and the normal functioning of the adrenal cortex. This problem is now being investigated by several techniques.

Our thanks are due to the John and Mary R. Markle Foundation for its support of this work, to Merck and Company, Inc., Rahway, New Jersey, for the crystalline vitamins used in the animal diets, and to Mead Johnson and Company, Evansville, Indiana, for a generous supply of amigen.

SUMMARY

The parenteral administration of physiological saline solution to dogs in blacktongue resulted in alleviation of the deficiency syndrome, and their lives were prolonged for as much as 180 days. All animals finally succumbed to the nicotinic acid deficiency but their state, *in extremis*, seldom resembled the classical picture of blacktongue. The results of analyses of the blood, urine, and tissues of normal dogs and dogs in acute blacktongue as well as in this more extensive nicotinic acid deficiency are presented and the significance of these findings is discussed.

BIBLIOGRAPHY

1. Elvehjem, C. A., Madden, R. J., Strong, F. M., and Woolley, D. W., *J. Biol. Chem.*, **123**, 137 (1938).
2. Goldberger, J., and Wheeler, G. A., *Pub. Health Rep., U. S. P. H. S.*, **43**, 172 (1928).
3. Warburg, O., and Christian, W., *Biochem. Z.*, **275**, 464 (1935).
4. von Euler, H., Albers, H., and Schlenk, F., *Z. physiol. Chem.*, **237**, 1 (1935).
5. Stannus, H. S., *Lancet*, **1**, 352 (1940).
6. Kohn, H. I., Klein, J. R., and Dann, W. J., *Biochem. J.*, **33**, 1432 (1939).
7. Dann, W. J., and Handler, P., *J. Nutrition*, **22**, 409 (1941).
8. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, **46**, 2066 (1924).
9. Benedict, S. R., *J. Biol. Chem.*, **68**, 759 (1926).
10. Van Slyke, D. D., and Stadie, W. C., *J. Biol. Chem.*, **49**, 1 (1921).
11. Joseph, N. R., and Stadie, W. C., *J. Biol. Chem.*, **125**, 795 (1938).
12. Elgart, S., and Harris, J. S., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 758 (1940).
13. Lu, G. D., *Biochem. J.*, **33**, 249 (1939).
14. Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
15. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
16. Dann, W. J., and Handler, P., *J. Biol. Chem.*, **140**, 201 (1941).
17. Kohn, H. I., *Biochem. J.*, **32**, 2075 (1938).
18. Handler, P., and Dann, W. J., *J. Biol. Chem.*, **140**, 739 (1941).

STUDIES ON THE HEMORRHAGIC SWEET CLOVER DISEASE

VIII. THE EFFECT OF 2-METHYL-1,4-NAPHTHOQUINONE AND L-ASCORBIC ACID UPON THE ACTION OF 3,3'-METHYLENEBIS(4-HYDROXYCOUMARIN) ON THE PROTHROMBIN TIME OF RABBITS*

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The purpose of this communication is to indicate the influence of 2-methyl-1,4-naphthoquinone and L-ascorbic acid on the hypoprothrombinemia produced by the anticoagulant and hemorrhagic agent 3,3'-methylenebis(4-hydroxycoumarin) in rabbits susceptible to the hemorrhagic sweet clover disease (1, 2). In the course of the isolation of the anticoagulant¹ from spoiled sweet clover hay (3) it was observed that the extent and duration of the induced hypoprothrombinemia were influenced by the food intake. Comparable concentrates from the spoiled hay gave more pronounced responses in fasted than in fed assay rabbits. This observation was subsequently confirmed with 3,3'-methylenebis(4-hydroxycoumarin) (4).

There are certain gross similarities between the syndrome resulting from the continued administration of 3,3'-methylenebis(4-hydroxycoumarin) and the hemorrhagic manifestations caused by a dietary deficiency of L-ascorbic acid and substances of the vitamin K class. These similarities, in conjunction with the similarities in the chemical structure of these substances, suggested that there might be an interrelationship in their modes of action, having a bearing on the maintenance of normal prothrombin levels or activity.

EXPERIMENTAL

Definite amounts of 3,3'-methylenebis(4-hydroxycoumarin) were fed standardized susceptible rabbits which were given supplements of 2-methyl-

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¹ The term anticoagulant is used in the general sense that 3,3'-methylenebis(4-hydroxycoumarin) is an agent, which after action *in vivo*, impairs or prevents the coagulation of blood. It does not affect the clotting power when added to blood or plasma *in vitro* (1, 3).

1,4-naphthoquinone or *l*-ascorbic acid, and the effect of the supplement in reducing the hypoprothrombinemia caused by the anticoagulant was measured. The technique of estimating the prothrombin time of the plasma, the method of selecting and standardizing the test animals, and the general procedure of following the hypoprothrombinemia through the use of 12.5 per cent plasma have already been fully described in previous papers (3, 4).

The rabbits used were in good physical condition, 2 to 3 years old, and weighed about 2.5 kilos. It should be emphasized that rabbits with approximately the same degree of susceptibility to 3,3'-methylenebis(4-hydroxycoumarin) were used. Unless otherwise stated they were fasted from 24 to 36 hours before each experiment and given only water thereafter until the normal prothrombin time was restored.

The 3,3'-methylenebis(4-hydroxycoumarin), the quinone, and the *l*-ascorbic acid were weighed into gelatin capsules and administered by mouth. A normal blood sample was taken at the time of the first feeding of the 3,3'-methylenebis(4-hydroxycoumarin) and at intervals thereafter until the normal prothrombin time was restored. Between experiments the rabbits were maintained for 7 to 10 days on a diet composed of oats 115 parts, corn 50 parts, bran 25 parts, linseed meal 10 parts, salt 3 parts, and U. S. No. 1 Extra Leafy Extra Green alfalfa hay *ad libitum*.

Unless otherwise indicated, the results are presented by curves showing the average changes in the prothrombin time of the 12.5 per cent plasmas of the various experimental groups when only 3,3'-methylenebis(4-hydroxycoumarin) was fed, and the average change when the same animals were fed an equivalent amount of the anticoagulant along with the quinone and the *l*-ascorbic acid. Because the selected susceptible animals show some variation in response to the anticoagulant (4), it was felt that the average response of the same group of animals should be employed to indicate the effects of the quinone and *l*-ascorbic acid.

Effect of Grain and Alfalfa Diet on Hypoprothrombinemia Induced by 3,3'-Methylenebis(4-hydroxycoumarin)—Curve A, Fig. 1, shows the average increase in prothrombin time of five rabbits which were fasted for 36 hours, fed 5 mg. of the anticoagulant, and given access only to water until the normal prothrombin time of the 12.5 per cent plasma was restored. Curve B shows the response induced by 5 mg. of the anticoagulant on the same rabbits when they were maintained on the grain and alfalfa diet over the entire period of the test. Curve C indicates the prothrombin times realized when the rabbits were maintained on the stock diet and given 5 mg. of the anticoagulant, 20 mg. of *l*-ascorbic acid, and 25 mg. of 2-methyl-1,4-naphthoquinone at the start and again on the 3rd day of this experiment.

It is apparent from Fig. 1 that the extent and duration of the hypopro-

thrombinemia induced by a 5 mg. dose of 3,3'-methylenebis(4-hydroxycoumarin) were markedly reduced when the animals were given access to the grain-alfalfa diet continuously, and completely prevented when 2-methyl-1,4-naphthoquinone and *l*-ascorbic acid were added to the diet.

Effect of 2-Methyl-1,4-naphthoquinone on Action of Anticoagulant—The action of substances in the vitamin K class has been under consideration since the early stages of our work on the hemorrhagic sweet clover disease. In the paper describing our bioassay (3) it was indicated that 2-methyl-1,-

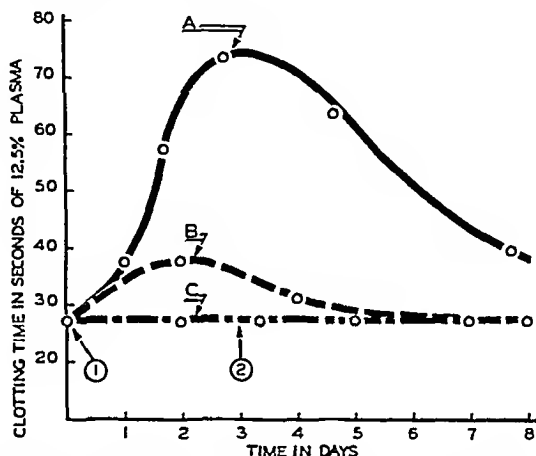


FIG. 1. The effect of a grain and alfalfa diet on the prothrombin time following the administration of 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin). Curve A represents the response of five rabbits when fasted for 36 hours before being fed the anticoagulant and given access to water only until the normal prothrombin time of 12.5 per cent plasma was restored; Curve B, the same animals maintained on a grain and alfalfa diet over the entire period of test; Curve C, the same rabbits maintained on the stock diet and given 20 mg. of *l*-ascorbic acid and 25 mg. of 2-methyl-1,4-naphthoquinone along with the anticoagulant at the start (Arrow 1) and on the 3rd day (Arrow 2).

4-naphthoquinone alone was ineffective both in preventing the hemorrhagic action of spoiled sweet clover hay and in hastening the restoration of normal prothrombin levels. The levels at which the quinone was fed in the initial studies were of the order which should have prevented symptoms of a dietary deficiency of vitamin K. After 3,3'-methylenebis(4-hydroxycoumarin) became available for more extensive trials, it was noted that a counteracting influence of 2-methyl-1,4-naphthoquinone could be detected, provided the antihemorrhagic substance was administered at high levels and the anticoagulant at low levels.

Ten rabbits were used in one experiment. Each rabbit was fed 3.0 mg. of 3,3'-methylenebis(4-hydroxyeoumarin) and the prothrombin times taken at intervals after feeding. This group of rabbits, after a rest period of 10 days, was fed the same amount of 3,3'-methylenebis(4-hydroxyeoumarin) simultaneously with large single feedings of 2-methyl-1,4-naphthoquinone. Curve A, Fig. 2, shows the average response of this group of rabbits to 3.0 mg. of 3,3'-methylenebis(4-hydroxyeoumarin). Curve B shows the average response when 30 mg. of 2-methyl-1,4-naphthoquinone were given along with 3.0 mg. of the anticoagulant. Curve C represents the effect of 3.0 mg.

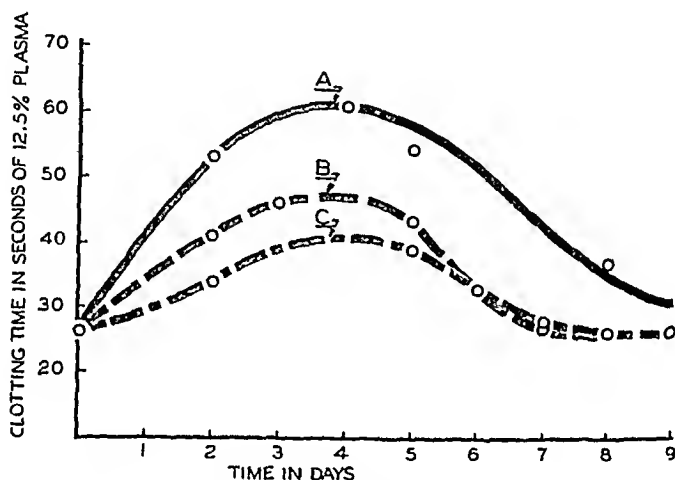


FIG. 2. The effect of 2-methyl-1,4-naphthoquinone on the prothrombin time following administration of 3,3'-methylenebis(4-hydroxyeoumarin). Curve A represents the response of ten rabbits given 3 mg. of anticoagulant alone; Curve B, that on 30 mg. of 2-methyl-1,4-naphthoquinone plus 3 mg. of the anticoagulant; Curve C, that on 3 mg. of anticoagulant when 30 mg. of 2-methyl-1,4-naphthoquinone were fed per day 3 days prior to and each day after the feeding of the anticoagulant.

of 3,3'-methylenebis(4-hydroxyeoumarin) on the same animals when they were fed 30 mg. of 2-methyl-1,4-naphthoquinone per day for 3 days prior to and each day after the feeding of the anticoagulant until the normal prothrombin time was restored.

These results show a moderate but definite alleviation of the extent and duration of the hypoprothrombinemia by the 2-methyl-1,4-naphthoquinone. However, it should be emphasized that very large levels of the quinone were necessary to counteract the effect of small amounts of 3,3'-methylenebis(4-hydroxyeoumarin). When the quinone was administered along with the anticoagulant in amounts comparable to those effective in al-

leviating a vitamin K deficiency, it was generally without effect on the action of the anticoagulant. This is in agreement with our original observation on the rabbit (3), and in the interim reports by other investigators have appeared that substantiate this. Butt, Allen, and Bollman (5), Bingham, Meyer, and Pohle (6), and Lehman (7) have reported that synthetic naphthoquinone preparations administered to dogs and man at the levels found effective for a deficiency of vitamin K did not shorten the prothrombin time previously lengthened by administration of 3,3'-methylenebis(4-hydroxycoumarin).

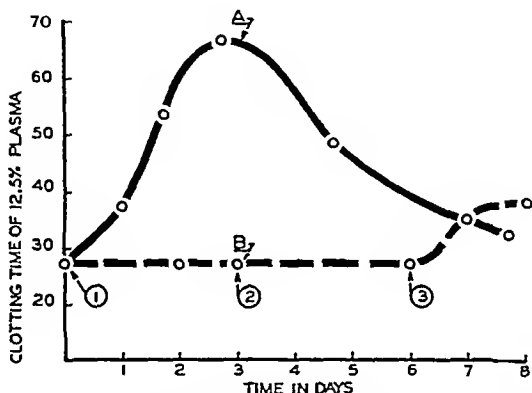


FIG. 3. The effect of *l*-ascorbic acid on the prothrombin time following the administration of 3,3'-methylenebis(4-hydroxycoumarin). Curve A represents the response (clotting time, measured in seconds) of fifteen rabbits when 3 mg. of the anticoagulant were fed; Curve B, that when 10 mg. of *l*-ascorbic acid in addition to 3 mg. of anticoagulant were fed at zero hour (Arrow 1) and on the 3rd day (Arrow 2). Arrow 3 represents the point at which 3 mg. of the anticoagulant alone were fed.

Effect of l-Ascorbic Acid on Action of Anticoagulant—When the effect of *l*-ascorbic acid was studied, it was found that most of the rabbits indicated as severe a hypoprothrombinemia when *l*-ascorbic acid was fed with the anticoagulant as when the anticoagulant was fed alone. Fifteen of 50 rabbits, however, showed no increase in the prothrombin time when 10 to 40 mg. of ascorbic acid were given simultaneously with 2.5 to 5 mg. of 3,3'-methylenebis(4-hydroxycoumarin).

Curve A, Fig. 3, shows the average increase in prothrombin time when 3.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) were fed to the fifteen animals mentioned above. Curve B indicates the protective or antagonistic action observed when 10.0 mg. of *l*-ascorbic acid were fed with 3.0 mg. of the anticoagulant at zero hour and again on the 3rd day. No increase in pro-

thrombin time was noted following the simultaneous administration of the two substances at the beginning and again on the 3rd day. But when 3.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) were fed *alone* on the 6th day, a slight rise in prothrombin time resulted. This was repeated on these fifteen animals five times with substantially the same effect.

In contrast to the above, certain rabbits in the original group could not be protected completely against the action of a single dose of 3.0 mg. of the anticoagulant, with levels of *l*-ascorbic acid as high as 75 mg.

Curve A, Fig. 4, is representative of individuals in which *l*-ascorbic acid exhibited no protective action, while Curve B is representative of indi-

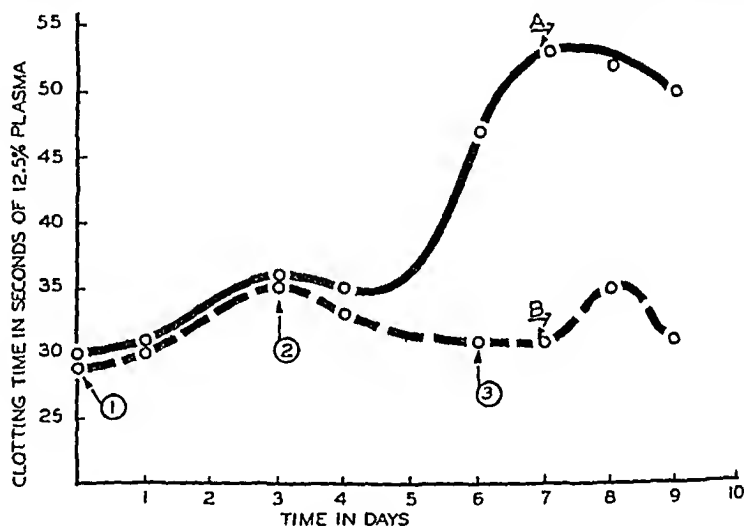


FIG. 4. The variation in the effect of *l*-ascorbic acid on the prothrombin time following the administration of 3,3'-methylenebis(4-hydroxycoumarin). Curve A represents individuals in which *l*-ascorbic acid had no protective action; Curve B, those protected. Arrow 1 indicates the point at which 2.5 mg. of anticoagulant were fed; Arrow 2, 2.5 mg. of anticoagulant and 15 mg. of *l*-ascorbic acid; Arrow 3, 2.5 mg. of anticoagulant.

viduals in which *l*-ascorbic acid was protective. At the beginning of the test 2.5 mg. of the anticoagulant were fed at zero hour. The usual increase in prothrombin time for these particular animals resulted. On the 3rd day a second 2.5 mg. dose of the anticoagulant was given along with 15 mg. of *l*-ascorbic acid. A continued increase in the prothrombin time is indicated in Curve A, while Curve B shows a downward trend toward normal clotting times. On the 6th day both individuals received a third 2.5 mg. dose of the anticoagulant, but no *l*-ascorbic acid, with the result that the prothrombin time was increased in both animals.

Effect of 2-Methyl-1,4-naphthoquinone and l-Ascorbic Acid on Action of

Anticoagulant—When 2-methyl-1,4-naphthoquinone and *l*-ascorbic acid were fed simultaneously with 3,3'-methylenebis(4-hydroxycoumarin) to fasted rabbits, the hypoprothrombinemia was markedly reduced in *all* of the animals. The following is representative: Five rabbits fed 2.5 mg. of the anticoagulant, after the usual 24 to 36 hour fast, showed an increase in the average prothrombin time from the normal value of 25 seconds, to 38 seconds after 48 hours, and 46 seconds after 72 hours. When 2.5 mg. of the anticoagulant, 20 mg. of the quinone, and 10.0 mg. of *l*-ascorbic acid were given simultaneously to the same animals, the average prothrombin time was 28 seconds after 48 hours and 33 seconds after 72 hours. These results are comparable to those represented by Curves A and C of Fig. 1.

Thus the antagonistic or protective action of 2-methyl-1,4-naphthoquinone and *l*-ascorbic acid against the anticoagulant action of 3,3'-methylenebis(4-hydroxycoumarin) is greatly increased when these substances are administered simultaneously to fasted rabbits.

DISCUSSION

The foregoing results indicate that *l*-ascorbic acid and 2-methyl-1,4-naphthoquinone at high levels both exert an antagonistic effect toward the prothrombin-reducing or inactivating properties of 3,3'-methylenebis(4-hydroxycoumarin) in rabbits. We do not at present know how these essential dietary factors counteract the action of the anticoagulant, nor, indeed, exactly how any of them exerts its characteristic physiological effects. The similarities in the gross pathological effects of deficiencies of *l*-ascorbic acid (8) or 2-methyl-1,4-naphthoquinone (9) and of the continuous administration of the anticoagulant might indicate that the action of 3,3'-methylenebis(4-hydroxycoumarin) may in part be due to disturbances in the metabolism of *l*-ascorbic acid and the antihemorrhagic quinone in the animal body.

The small quantity of 3,3'-methylenebis(4-hydroxycoumarin) which will produce a prolongation in prothrombin time² suggests that the anticoagulant most likely acts by blocking some enzyme system. The inactivation of enzyme systems by certain compounds structurally related to their substrates, the structural similarities of anticoagulant 4-hydroxycoumarins³ to substances possessing vitamin K activity, and the antagonistic action of 2-methyl-1,4-naphthoquinone toward this anticoagulant lend support to the suggestion that this anticoagulant and 2-methyl-1,4-naphthoquinone act through a common system. The results suggest that *l*-ascorbic acid is involved in this system. However, it should be pointed out that the levels of 2-methyl-1,4-naphthoquinone and *l*-ascorbic acid neces-

² It should be emphasized that the action of a single 0.20 mg. dose of the anticoagulant is detectable in selected rabbits.

³ Based on a study of the relationship of chemical structure to anticoagulant action of 4-hydroxycoumarins. To be published later.

sary to counteract the action of the anticoagulant are considerably larger than those required to avoid a dietary deficiency. A physiological relationship between vitamins K and C has been reported by Tomaszewski and Engel (10) and Campana (11). That both vitamins are somehow involved in the action of the anticoagulant in other species has been subsequently observed in this laboratory in the rat (12)⁴ and the guinea pig (13).

SUMMARY

1. Feeding susceptible rabbits a grain mixture and alfalfa hay reduced the anticoagulant action of single 2.5 to 5.0 mg. doses of 3,3'-methylenebis(4-hydroxycoumarin) as measured by the increase in the clotting time of 12.5 per cent plasma.

2. The simultaneous oral administration of 2-methyl-1,4-naphthoquinone and *l*-ascorbic acid at high levels along with 2.5 to 5.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) either drastically reduced or completely nullified the anticoagulant action in all the rabbits tested.

3. The oral administration of 2-methyl-1,4-naphthoquinone at high levels along with 2.5 to 5.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) reduced the anticoagulant action in all the rabbits tested.

4. The oral administration of *l*-ascorbic acid at high levels along with 2.5 to 5.0 mg. of 3,3'-methylenebis(4-hydroxycoumarin) prevented the usual increase in the prothrombin time in some of the rabbits. In contrast, in certain rabbits, *l*-ascorbic acid alone exhibited no antagonistic action to the anticoagulant.

BIBLIOGRAPHY

1. Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **138**, 21 (1941).
2. Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.*, **138**, 513 (1941).
3. Campbell, H. A., Smith, W. K., Roberts, W. L., and Link, K. P., *J. Biol. Chem.*, **138**, 1 (1941).
4. Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *J. Biol. Chem.*, **142**, 941 (1942).
5. Butt, H. R., Allen, E. V., and Bollman, J. L., *Proc. Staff Meetings Mayo Clin.*, **16**, 388 (1941).
6. Bingham, J. B., Meyer, O. O., and Pohle, F. J., *Am. J. Med. Sc.*, **202**, 563 (1941).
7. Lelham, T., *Lancet*, **1**, 318 (1942).
8. Presnell, A. K., *J. Nutrition*, **8**, 69 (1934).
9. Dam, H., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **2**, 285 (1942).
10. Tomaszewski, W., and Engel, C., *Z. Vitaminforsch.*, **9**, 238 (1939).
11. Campana, C., *Boll. Soc. ital. biol. sper.*, **16**, 157 (1941).
12. Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition*, **23**, 589 (1942).
13. Sullivan, W. R., Dissertation, University of Wisconsin (1942).

⁴ Baumann, C. A., Field, J. B., Overman, R. S., and Link, K. P., in preparation.

THE NON-SPECIFICITY OF THIAMINE IN FAT SYNTHESIS*

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According to a current theory, one function of thiamine is the formation of fat (1). Increases in body fat which have been observed after thiamine administration to deficient animals have been cited (2) as substantiating evidence for the theory, and a study has been made of the chemical composition of the "fatty acids synthesized by the action of thiamine" (3). In the experiments cited, thiamine feeding was preceded by a 2 to 3 week period of depletion on a diet deficient in the vitamin B complex. Whether the animals were actually depleted of factors other than thiamine was not determined. Thus, the question arises whether the observed fat increases were indicative of a specific action of thiamine *per se*, or whether they were primarily reflective of a general improvement in the nutritive state of the animal.

The present study deals with body fat increases in response to various H₂O-soluble supplements after long periods of depletion on two low fat diets, the first of which was deficient primarily in thiamine and the second ample in thiamine but deficient in other essential factors.

EXPERIMENTAL

The two basal diets used were Diet AY, which contained autoclaved yeast, and Diet V which has been used in this laboratory for acrodynia studies and which contained crystalline thiamine and riboflavin¹ (Table I).

For the studies with Diet AY, 50 gm. rats from our stock colony were used. When depleted of their thiamine reserves, as evidenced by stationary body weight over a period of 1 week, they were divided into three groups. Group 1 was taken at once for an analysis of total fatty acids. Group 2 received 10 γ of thiamine chloride daily for 3 weeks and the basal diet *ad libitum*. Group 3 also received 10 γ of thiamine chloride daily, but the intake of the basal diet was so restricted that body weights were held stationary. Groups 2 and 3 were analyzed at the end of a 3 week period.

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¹ The riboflavin was generously furnished by Merck and Company, Inc.

For the studies with Diet V, 40 gm. rats from Diet P, consisting chiefly of potato meal (5), were used. On Diet V, acrodynia developed during the 5th week. Animals with dermal indices of 5 to 6 (6) were divided into four groups (Groups 4 to 7). Group 4 was used immediately for the fat analysis. Group 5 received 50 γ of thiamine chloride daily in addition to the 10 γ which were given with Diet V. Group 6 received 200 mg. of rice bran concentrate (vitab). Groups 5 and 6 were fed the basal diet *ad libitum*. Group 7 received 200 mg. of vitab but consumption of the basal diet was restricted as with Group 3. Groups 5, 6, and 7 were analyzed after having received the supplements for 3 weeks.

TABLE I
Diets and Body Weight Changes Prior to Fat Analysis

Group No.	Basal diet*	Daily supplement	No. of rats	At time of analysis		
				Age	Mean body weight	Mean 21 day gain
				wks.	gm.	gm.
1	AY	None	5	7	76	
2	"	Thiamine, 10 γ	6	10	127	52
3	" (restricted)	" 10 γ	5	10	72	0
4	V	None	6	9	56	
5	"	Thiamine, 50 γ	4	12	60	2
6	"	Vitab, 200 mg.	4	12	100	40
7	" (restricted)	" 200 "	8	12	56	0

* Diet AY (4) consisted of casein 18, Salts 40 (Steenbock and Nelson) 4, autoclaved yeast 8, agar 2, dextrinized starch 76, and cod liver oil 2. Diet V (5) consisted of casein 18, salts (Wesson) 4, glucose 78, supplemented daily with carotene 10 γ , calciferol 5 γ , thiamine chloride 10 γ , riboflavin 20 γ . Animals in Groups 3 and 7 were weighed daily and food intake restricted to prevent gains. Labor for the purification of the casein was furnished by the Works Progress Administration.

In the fat analyses, total fatty acids of the liver and the remaining portions of the carcass were determined separately. The procedure has been described (7).

Results

The changes in body weight which occurred during the 3 week period are shown in Table I. The data from the fat analyses are summarized in Table II. The results obtained with the thiamine-deficient animals (Diet AY) were similar to those reported by McHenry and Gavin (2). Group 1, which was representative of the animals at the time supplements were begun, contained 2.3 per cent fat with an iodine value of 91. Group 2, which had shown a typical growth response of 52 gm. during a 3 week period

of thiamine supplement, had increased its fat content to 7.0 per cent. Group 3 which received the same supplement of thiamine, but due to restricted food intake had not increased in body weight during the period, evidenced no increase in fat.

The results obtained with acrodynic animals were almost identical with those obtained with thiamine-deficient animals. Group 4, which had received 10 γ of thiamine daily but was deficient in other factors including pyridoxine and pantothenic acid (6), contained 2.2 per cent of body fat or approximately the same percentage as the thiamine-deficient animals. Similar animals, Group 5, showed no appreciable response to a supplement of 50 γ of thiamine. However, 200 mg. of vitab, which supplied approximately the same amount of thiamine and in addition other factors which

TABLE II

Effect of Diet on Fatty Acid Content of Tissues

The weight of the fatty acids is given in gm.

Group No.	Diet*	Liver fatty acids			Carcass fatty acids		
		Weight, average	Per cent	Iodine value	Weight, average	Per cent	Iodine value
1	AY	0.03	2.0	149	1.7	2.3	91
2	" + thiamine	0.16	1.9	145	8.3	7.0	74
3	" (restricted) + thiamine	0.07	1.9	142	1.6	2.4	88
4	V	0.07	2.3	101	1.2	2.2	81
5	" + thiamine, 50 γ	0.09	2.9	121	1.4	2.4	81
6	" + vitab	0.13	2.6	101	5.8	6.2	67
7	" (restricted) + vitab	0.09	3.0	99	1.4	2.6	76

* See Table I.

cured the acrodynia, produced a gain of 40 gm. with a concurrent increase in body fat to 6.2 per cent. With restricted food intake, vitab supplements produced no significant change in fat (Group 7).

As indicated by the iodine values, the fatty acids from deficient animals had a relatively high degree of unsaturation. After correction of deficiencies on either diet the degree of unsaturation was reduced.

No appreciable changes were revealed in the liver fatty acids following supplements on either diet. Fatty livers were not observed after 3 weeks on the thiamine supplement.

DISCUSSION

The results show that the fat changes observed in thiamine deficiency are observed in other deficiencies as well, even in the presence of adequate amounts of thiamine. For example, on two deficient diets the fat content

of the depleted rats fell to the low levels of 2.2 to 2.3 per cent respectively, and while the thiamine-deficient group responded to thiamine with a 5-fold increase in total fat, the acrodynic group which was already receiving adequate thiamine likewise responded to the rice bran concentrate with a 5-fold increase in total fat. That thiamine was not directly involved in the latter case was shown by the lack of response to additional thiamine. Furthermore, the other specific factors were able to effect responses in such animals, as shown in Table III from data taken from an earlier report (7). Pyridoxine fed with pantothenic acid produced an increase in total fat, but either supplement fed alone was ineffective.

Thus it has been shown that on a diet high in carbohydrate and low in fat a normal deposition of tissue fat was prevented by a deficiency of pyridoxine or pantothenic acid as well as by a deficiency of thiamine. In each case, correction of the deficiency resulted in a substantial deposition of fat when the intake of basal diet was not restricted. It would seem

TABLE III
Fat Increases with Pyridoxine and Pantothenic Acid in Multiple Deficiency

Supplement to Diet V	Carcass fatty acids	Liver fatty acids
	<i>per cent</i>	<i>per cent</i>
Pantothenic acid, 50 γ	2.3	2.6
Pyridoxine, 20 γ	2.6	2.0
Pantothenic acid, 50 γ , + pyridoxine, 20 γ	5.0	3.9

likely that a similar effect could be demonstrated for any vitamin or other dietary essential, a deficiency of which seriously diminishes food consumption and thereby limits growth and development. In view of the results presented, it is evident that the production of fat in the animal body should not be considered as a function of any single dietary essential unless it has been proved to the exclusion of others.

The data presented are not contradictory to those of McHenry and his collaborators (2, 3). Reinterpretation of their results in the light of present findings reveals that they were dealing primarily with a thiamine deficiency rather than a multiple deficiency. This follows, since their animals showed substantial increases in fat when fed thiamine alone. While their diet was deficient in the entire vitamin B complex, thiamine must have been the only vitamin depleted sufficiently to prevent growth at the end of 2 or 3 weeks. The conditions of their experiments were essentially the same as ours with Diet AY. In theirs, the B vitamins other than thiamine were supplied from the undepleted reserves of the animals; in ours, they were supplied continuously in the diet.

The true effects of factors other than thiamine on fat synthesis are revealed only when they too serve for the complete correction of a deficiency. In our experiment this was made possible by feeding thiamine, thereby extending the survival period and allowing depletion with respect to other essentials. Conceivably, depletion of other factors as well as thiamine could be accelerated by increasing their dietary requirement. There is some evidence that acrodynia is intensified on high protein diets (8, 9). Should this prove indicative of an increased requirement for pyridoxine on high protein diets, it would provide a basis for reinterpreting the importance of pyridoxine in the synthesis of fat from protein (10).

SUMMARY

Thiamine did not prevent rapid losses of total fat in rats on a diet deficient in other B vitamins.

Thiamine was not more effective than other B vitamins in increasing the total fat content of rats.

The effectiveness of a supplement in restoring normal fat synthesis in deficient rats is apparently determined by the completeness with which it supplies essentials lacking in the body tissues as well as in the basal diet.

BIBLIOGRAPHY

1. McHenry, E. W., *Science*, **86**, 200 (1937)
2. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **125**, 653 (1938).
3. Longenecker, H. E., Gavin, G., and McHenry, E. W., *J. Biol. Chem.*, **134**, 693 (1940)
4. Kemmerer, A. R., and Steenbock, H., *J. Biol. Chem.*, **103**, 353 (1933). Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **56**, 355 (1923)
5. Quackenbush, F. W., Platz, B. R., and Steenbock, H., *J. Nutrition*, **17**, 115 (1939). Wesson, L. G., *Science*, **75**, 339 (1932)
6. Quackenbush, F. W., Steenbock, H., Kummerow, F. A., and Platz, B. R., *J. Nutrition*, in press
7. Quackenbush, F. W., and Steenbock, H., *J. Nutrition*, in press
8. György, P., and Eckardt, R. E., *Biochem. J.*, **34**, 1143 (1940)
9. Conger, T. W., and Elvehjem, C. A., *J. Biol. Chem.*, **138**, 560 (1941).
10. McHenry, E. W., and Gavin, G., *J. Biol. Chem.*, **138**, 471 (1941)

TOCOPHEROL AND THE STABILITY OF CAROTENE*

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That carotene is less effective as a source of vitamin A when fed in pure solvents than in crude oils has been observed by many workers. First it was found that carotene dissolved in ethyl oleate was inactive as a source of vitamin A (1). This inactivity was later attributed to oxidation (2). Following the demonstration that autoxidation of lard could be prevented by polyphenols (3), hydroquinone was reported to stabilize carotene in ethyl oleate or ethyl laurate solution (4). Thereafter it became common practice to use hydroquinone as a stabilizer (5-8).

However, even with added hydroquinone, carotene in certain oils was found inefficient as a source of vitamin A. Kraybill and Shrewsbury (9) reported that carotene was one-fourth as active in butter fat which had been treated with charcoal as in cottonseed oil. The addition of hydroquinone prevented the destruction of carotene in storage but did not increase its biological potency. In butter fat which had been treated with Lloyd's reagent carotene remained stable at 40°, but it was only one-half as potent biologically as carotene in cottonseed oil. It was suggested (9) that "Lloyd's reagent may have removed a factor which supplemented the vitamin A activity of the carotene." Lease *et al.* (10) found that carotene was unstable at 0° in butter fat which had been treated with Lloyd's reagent. Coward (11) attempted to explain the differences in potency of carotene in various solvents by differences in utilization of carotene but concluded that the causes must be sought elsewhere. Lathbury and Greenwood (12) reported that carotene gave poorer responses when administered in coconut oil with or without quinol than in linseed oil. They stated that an oil which allowed the best growth might contain some factor necessary to supplement highly purified preparations of vitamin A or its precursors.

Sherman (13) presented further evidence for the need of an additional substance when carotene was given in purified oils. Symptoms of vitamin A deficiency were not cured with low levels of carotene in methyl linolate,

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but were cured with the same supplement in soy bean oil or in methyl linolate plus soy bean oil. Since fecal excretion did not account for the differences, linoleic ester was believed to have interfered with the metabolism of the carotene, which reaction was preventable by some constituent of soy bean oil.

Quackenbush, Cox, and Steenbock (14) found that tocopherol promoted a response to carotene and concluded that the tocopherol functioned in the gastrointestinal tract by virtue of its antioxidant properties. Sherman (15) suggested that tocopherol was concerned with the utilization of carotene rather than with its protection from oxidation.

The present work throws further light on this problem. It deals with the relation of tocopherol and other antioxidants to the biological effectiveness of carotene and correlates the results with the stability of carotene *in vitro*.

EXPERIMENTAL

Ethyl linolate was prepared from corn oil (Mazola) by a procedure similar to that of Rollett (16). The pure tetrabromide (m.p. 115°) was debrominated with zinc and HCl in absolute alcohol and the product was then distilled under reduced pressure (0.5 mm.) and immediately sealed in glass ampules under a high vacuum. All samples were shown to be peroxide-free when used.

β -Carotene was prepared from a commercial product by chromatographic adsorption on MgO and recrystallization from carbon disulfide and petroleum ether. Its concentration in oil solutions was determined by diluting with freshly distilled chloroform and measuring the spectral absorption at 440 m μ with an Evelyn photoelectric colorimeter. This method was sensitive to 5 γ of carotene.

For the biological assays the carotene solutions were prepared weekly, except when stated otherwise. 1.5 gm. of the ethyl linolate or other solvent were weighed into a 25 cc. ampule and β -carotene was added in petroleum ether or peroxide-free ethyl ether. The solvents were removed under reduced pressure with agitation of the flask in a water bath at 37° for 10 minutes. The supplements were stored under nitrogen at 0°, except when the vials were opened for feeding.

The basal diet (Diet 36) (17) used for the biological assays was a fat-free diet consisting of casein (alcohol-extracted) 18 gm., glucose (cerealose) 75 gm., Wesson salts¹ 4 gm., vitab (a rice bran concentrate) 3 gm., and riboflavin² 170 mg. In addition each rat received weekly 25 γ of calciferol dissolved in 1 drop of hydrogenated coconut oil.

¹ Wesson, L. G., *Science*, 75, 339 (1932).

² The riboflavin, α -tocopherol, vitamin K₁, and 2-methyl-1,4-naphthoquinone were generously supplied by Merek and Company, Inc.

In preparation for the assays, rats 4 weeks of age weighing between 45 and 55 gm. were placed in individual cages and given basal Diet 36 *ad libitum*. Between the 4th and 5th weeks, they began to lose weight and their eyelids were bare and slightly swollen. They were used for assay when their body weight had remained constant or declined for 1 week. Supplements were evaluated in terms of changes in weight. When a majority of the animals in a group gained, only those which survived the 21 day period were included in calculation of the averages. However, when less than 50 per cent gained, those which did not survive the full period were also included, in which case the gain or loss was calculated on the last weight recorded before death.

TABLE I
Effect of Solvent on Potency of Carotene Fed at 5 γ Daily

Group No.	Solvent	No. of rats	Change in weight per rat in 21 days
			gm.
1	No supplement	4	-12
2	Ethyl linolate	8	-8
3	" " (fresh solution daily)	6	-17
4	" " + 0.02% hydroquinone	8	-11
5	Soy bean oil (fresh)	13	54
6	" " " (peroxide No. 40)	4	40
7	Ethyl linolate + soy bean oil	13	55
8	" " + 1% of 180° distillate from soy bean oil	7	35

Results

Protective Substance in Soy Bean Oil—The administration of 5 γ of carotene per day in 1 drop of ethyl linolate failed to restore growth or to cure ophthalmia even when the solution was prepared daily and fed immediately (Table I). When hydroquinone (0.02 per cent) was mixed with the linolate, the carotene was stabilized in storage and peroxides did not develop, but when it was fed the rats continued to lose weight (Group 4). However, the same amount of carotene in soy bean oil, whether fresh or oxidized to a peroxide number of 40, enabled the animals to gain weight. Soy bean oil therefore contained a protective substance, for which 0.02 per cent hydroquinone was not a substitute.

Molecular Distillation of Protective Substance—The protective substance was distillable in a cyclic molecular still. 108 gm. of refined soy bean oil when distilled at 100° for 1 hour, then at 180° for 4 hours, gave 1.64 gm. of solid distillate. This distillate diluted to a concentration of 1 per cent in linolate promoted a biological response to carotene.

Previous studies of this laboratory (18) had revealed that tocopherols distil quantitatively from soy bean oil under the conditions employed in the above experiment, and since the tocopherols were known to inhibit the uptake of oxygen by lard (19) it was assumed that the stabilizing agent in the distillate might be the tocopherols. Additional constituents of soy bean oil which would be expected to distil include vitamin K₁ (20), sterols (21) and other inhibitols (22).

Stabilization of Carotene in Vitro—Tests *in vitro*, to determine whether the above materials would protect carotene against oxidation in linolate solution, were made as follows: The desired quantity of the test substance and 125 γ of β -carotene in petroleum ether solution were placed in a small test-

TABLE II
Comparative Effects of Various Substances on Stability of Carotene in Ethyl Linolate in Vitro

Substance (15 γ)	Per cent of initial carotene remaining after 48 hrs. at	
	25°	37°
None .. .	0	0
α -Tocopherol (dl, synthetic)		76
Soy bean oil distillate (180°)*		96
" " " *		76
Crystalline sterols from soy bean oil distillate ...	0	0
Vitamin K ₁ (synthetic)	9	0
2-Methyl-1,4-naphthoquinone	14	0
Antioxidant concentrate (commercial)	18	0
Pyrogallol	100	100
Hydroquinone	98	74
Catechol	85	52
Guaiacol		0

* Added in amounts equivalent to 15 γ of α -tocopherol as determined photometrically.

tube (14 \times 100 mm.). The solvent was removed under reduced pressure and 500 mg. of fresh ethyl linolate were weighed into the tube at once. After an initial determination of carotene the tube was placed in a water bath at constant temperature in diffuse laboratory light and open to the air. Carotene determinations were made at intervals up to 48 hours.

When no antioxidant was added, the carotene disappeared completely within 48 hours at 25° as well as at 37° (Table II). However, 15 γ of α -tocopherol² (0.003 per cent) afforded 76 per cent protection at 37°. The distillate from soy bean oil or the original soy bean oil, when added in quantity sufficient to furnish 15 γ of tocopherol as determined photometrically (18), also protected the carotene. Of the other substances tested,

only pyrogallol, hydroquinone, and catechol protected carotene effectively at 37°.

Biological Potency of Preparations—In the animal assays α -tocopherol proved to be highly potent (Table III). Between 0.01 and 0.03 per cent or 2 to 6 γ per rat daily were sufficient to protect the carotene when administered in ethyl linolate.

The distillate from soy bean oil fed in amounts supplying the same levels of tocopherol, determined by photometric analysis, gave a slightly

TABLE III

Growth Responses to 5 γ of Carotene in Ethyl Linolate with Added Substances

Group No.	No. of rats	Substance added	Amount added	Change in weight per rat in 21 days
			per cent	gm.
9	19	None		-8
10	10	α -Tocopherol (synthetic)	0.10	36
11	5	" "	0.03	34
12	4	" "	0.02	16
13	4	" "	0.01	20
14	5	" "	0.003	-1
15	6	Soy bean distillate*	0.10	61
16	6	" " "	0.03	54
17	6	" " "	0.02	41
18	6	" " "	0.01	40
19	5	" " "	0.005	6
20	11	Vitamin K ₁ (synthetic)	0.10	-4
21	4	" " "	0.02	-5
22	6	2-Methyl-1,4-naphthoquinone	0.02	-15
23	6	Catechol	0.02	-5
24	13	Pyrogallol	0.02	-8
25	5	Guaiacol	0.02	-14
26	5	Hydroquinone	1.00	38
27	6	"	0.10	19
28	6	"	0.02	4

* The amounts added refer to the tocopherol content as determined photometrically.

greater response than that obtained with a corresponding amount of α -tocopherol. However, this difference is of doubtful significance, since the two substances were assayed separately with an interval of several months. Furthermore, the animals used in assaying the α -tocopherol had a high incidence of respiratory disorder, while those used for the distillate were relatively free from this handicap. Vitamin K was ineffective in the biological tests as well as *in vitro*.

The simple polyphenols did not show high protective action for carotene

in the biological tests as they did in the *in vitro* tests. In concurrent assays hydroquinone proved approximately 1/100 as effective as tocopherol supplied in the form of soy bean distillate (Groups 18 and 26). 0.02 per cent catechol seemed to give some protection in preliminary tests (14) but this was not confirmed in later experiments.

Since the polyphenols are more soluble in water than in fat, it was surmised that their low protective power in the biological tests was due to their

TABLE IV
Effect of Water Extraction upon Stabilization of Carotene (37°)

Antioxidant added		Treatment prior to incubation	Per cent of initial carotene remaining after 48 hrs
	γ		
None		None	0
Hydroquinone	15	"	97
"	15	Extracted	0
"	100	None	100
"	100	Extracted	0
α -Tocopherol	15	None	92
"	15	Extracted	95

TABLE V
Protective Action of Hydroquinone on Tocopherol

Sam- ple	Antioxidant added		Per cent of initial carotene remaining after 168 hrs	Treatment after 168 hrs	Per cent of initial carotene remaining after 336 hrs
		γ			
A	Tocopherol	15	21	None	0
B	" + hydroquinone	15 100	93	"	91
C	Tocopherol + hydroquinone	15 100	95	Extracted	40
D	Hydroquinone	100	96	"	0*

* The carotene was completely destroyed within 48 hours after extraction

extraction from the ethyl linolate by the aqueous fluids of the gastrointestinal tract. To examine this possibility, the effect of aqueous extraction upon the stability of carotene *in vitro* was studied. 1 gm. samples of carotene-linolate solutions containing hydroquinone or tocopherol were extracted twice with a 10-fold amount of water by shaking for 30 minutes in an atmosphere of nitrogen in 50 cc. bottles. The results (Table IV) show that extraction with water destroyed the effectiveness of hydroquinone but not of tocopherol.

Hydroquinone was found to stabilize tocopherol as well as carotene *in vitro*. Solutions of 125 γ of carotene in 500 mg. of ethyl linolate were treated with (Sample A) 15 γ of tocopherol, (Samples B and C) 15 γ of tocopherol plus 100 γ of hydroquinone, and (Sample D) 100 γ of hydroquinone, respectively. After 168 hours the carotene content of each sample was determined. Samples C and D were then extracted with water, simultaneously. After a second 168 hour period carotene was again determined. The results are shown in Table V. Since the action of hydroquinone was interrupted in Samples C and D by extraction with water, the greater stability of Sample C over Sample D at 336 hours must have been due to tocopherol. Furthermore, since as much carotene remained in Sample C at 336 hours as in Sample A at 168 hours, the tocopherol in Sample C had apparently been stabilized for 168 hours by the hydroquinone.

DISCUSSION

The data establish that α -tocopherol is highly effective both in preventing autoxidation of carotene *in vitro* and in promoting a biological response to carotene in ethyl linolate. They indicate, further, that the tocopherols are responsible for at least a part of the protective action of soy bean oil, since (a) both α -tocopherol and the protective substance of the soy bean oil distilled under similar conditions, and (b) in both the biological and the *in vitro* tests small amounts of α -tocopherol showed approximately the same potency as the same amounts of soy bean tocopherols. Whether soy bean oil contains other compounds which protect carotene but behave differently from tocopherols in a molecular still remains to be determined.

The evidence indicates that in promoting a biological response to carotene the tocopherol functions as an antioxidant in the gastrointestinal tract rather than as a vitamin regulating some phase of metabolism in the tissues. Both α -tocopherol and hydroquinone are antioxidants for carotene *in vitro* and both are capable of promoting a biological response to carotene. The amount of tocopherol required to protect carotene in feeding tests is only slightly greater than that required for protection of carotene *in vitro*. Further evidence for the antioxidant rôle of tocopherol is presented in a recent report by Sherman (23) that carotene was destroyed in the tract in the absence of tocopherol.

The comparative ineffectiveness of the polyphenols as stabilizers for carotene in feeding studies is not in accord with current belief and practice. Olcovich and Mattill (4) reported that ethyl laurate and ethyl oleate became suitable vehicles for the administration of carotene after the addition of hydroquinone, but it is to be noted that their conclusions were based primarily on studies *in vitro*. Close examination of their biological data fails to reveal a decisive demonstration that hydroquinone protected

carotene. The results of several other workers (8, 9, 12, 24) furnish evidence that oils lacking in natural antioxidants are not suitable vehicles for carotene even after the addition of 0.01 to 0.02 per cent of hydroquinone.

A different situation is encountered when hydroquinone is added to an oil containing small amounts of tocopherol. Our experiments indicate that hydroquinone is capable of protecting tocopherol from autoxidation *in vitro*, thereby preserving the latter for protection of the carotene in the tract. The literature reveals biological evidence for this with vitamin A. Huston *et al.* (25) found that butter fat and cod liver oil which were placed in the light for 50 to 56 days at 37° remained curative of vitamin A deficiency only when hydroquinone was added before the exposure. Turner (26) and Jones and Christianson (27) made similar observations. That substances other than hydroquinone may act similarly is indicated by the report of Golumbic and Mattill (28) that the antioxidant activity of tocopherol was markedly augmented by ascorbic acid.

From the evidence presented it is apparent that for *biological* studies the protection of carotene or vitamin A in an oil by hydroquinone is contingent upon the presence of tocopherol or its equivalent as some other lipophilic antioxidant. It is probable that many discrepancies which have been reported with respect to responses to carotene or vitamin A supplements can be explained on this basis. Those who have claimed no differences in effectiveness of carotene in different oils worked with oils which contained natural antioxidants. The apparently conflicting results with respect to biological activity of carotene in oils treated with adsorbents (9, 10) are probably attributable to differences in the degree to which natural antioxidants were removed by adsorption. While emphasis is here placed upon the importance of antioxidants, it should be recognized that other factors such as metal contaminants and peroxides in the diet are also involved. Much is still to be learned concerning the activity of peroxides, since it was revealed in the present study that tocopherol can stabilize carotene even in the presence of relatively high concentrations of peroxide. The relationship between tocopherol and peroxide formation will be dealt with in a later communication.

SUMMARY

Daily supplements of 5 γ of carotene in ethyl linolate failed to produce growth in young rats deficient in vitamin A. When a distillate from soy bean oil was given simultaneously, growth resulted. The protective factor in the distillate was apparently tocopherol, since equivalent amounts of synthetic α -tocopherol and soy bean tocopherol (determined photometrically) produced a similar response. α -Tocopherol was effective at concentrations of 0.01 to 0.03 per cent (2 to 6 γ daily). Vitamin K₁, pyrogallol, catechol, and hydroquinone were inactive at 0.02 per cent. However, hydroquinone gave a good response at 1.00 per cent.

α -Tocopherol and hydroquinone were equally effective antioxidants for the carotene in linolate *in vitro*. However, after extraction of the solutions with water, the carotene remained stable only in the tocopherol-treated samples. Hence, the low protective power of hydroquinone in the animal tests was probably due to its extraction from the lipid phase in the gastrointestinal tract.

Hydroquinone protected tocopherol from autoxidation in linolate solution *in vitro*, thus sparing the tocopherol for the protection of carotene in the tract.

It appears that the problem of instability of carotene in oil solutions exposed to oxygen can be solved by the use of lipophilic antioxidants such as the tocopherols.

BIBLIOGRAPHY

1. Duliere, W., Morton, A. R., and Drummond, J. C., *J. Soc. Chem. Ind.*, 48, 319 (1929).
2. Drummond, J. C., Ahmad, B., and Morton, A. R., *J. Soc. Chem. Ind.*, 49, 291 T (1930).
3. Mattill, H. A., *J. Biol. Chem.*, 90, 141 (1931).
4. Olcovich, H. S., and Mattill, H. A., *J. Biol. Chem.*, 91, 105 (1931).
5. Baumann, C. A., and Steenbock, H., *J. Biol. Chem.*, 101, 561 (1933).
6. McDonald, F. G., *J. Biol. Chem.*, 103, 455 (1933).
7. Turner, R. G., and Lowe, E. R., *J. Infect. Dis.*, 52, 102 (1933).
8. Dyer, F. J., Key, K. M., and Coward, K. H., *Biochem. J.*, 28, 875 (1934).
9. Kraybill, H. R., and Shrewsbury, C. L., *J. Nutrition*, 11, 103 (1936).
10. Lease, E. J., Lease, J. G., Steenbock, H., and Baumann, C. A., *J. Nutrition*, 17, 91 (1939).
11. Coward, K. H., *Biochem. J.*, 30, 1878 (1936).
12. Lathbury, K. C., and Greenwood, G. N., *Biochem. J.*, 28, 1665 (1934).
13. Sherman, W. C., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, 133, p. lxxxix (1940).
14. Quackenbush, F. W., Cox, R. P., and Steenbock, H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, 140, p. civ (1941).
15. Sherman, W. C., *Proc. Soc. Exp. Biol. and Med.*, 47, 199 (1941).
16. Rollett, A., *Z. physiol. Chem.*, 62, 410 (1909).
17. Quackenbush, F. W., Kummerow, F. A., and Steenbock, H., *J. Nutrition*, in press.
18. Quackenbush, F. W., Gottlieb, H. L., and Steenbock, H., *Ind. and Eng. Chem.*, 33, 1276 (1941).
19. Olcott, H. S., and Emerson, O. H., *J. Am. Chem. Soc.*, 59, 1003 (1937).
20. Almquist, H. J., *J. Biol. Chem.*, 120, 635 (1937).
21. Hickman, K. C. D., *Ind. and Eng. Chem.*, 32, 1451 (1940).
22. Bradway, E. M., and Mattill, H. A., *J. Am. Chem. Soc.*, 56, 2405 (1934).
23. Sherman, W. C., *Federation Proc.*, 1, pt. 2, 134 (1942).
24. Hume, E. M., and Smedley-MacLean, I., *Lancet*, 1, 290 (1930).
25. Huston, R. C., Lightbody, H. D., and Ball, C. D., Jr., *J. Biol. Chem.*, 79, 507 (1928).
26. Turner, R. G., *J. Biol. Chem.*, 105, 443 (1934).
27. Jones, W. S., and Christianson, J., *J. Am. Pharm. Assn.*, 24, 465 (1935).
28. Golumbic, C., and Mattill, H. A., *J. Am. Chem. Soc.*, 63, 1279 (1941).

DIETARY CHLORIDE DEFICIENCY AND ALKALOSIS IN THE RAT

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Signal advances in the knowledge of the biochemical functions of the different mineral elements have been made in recent years through studies of the effects of diets deficient only in a single element upon the animal organism. With certain of the mineral elements normally present in great abundance in the diet, notably potassium (1-3) and sodium (4-6), the degree of dietary deficiency must be very severe to bring out the characteristic effects of their deprivation.

Few experiments have been carried out on the effect of chloride deficiency and these have heretofore yielded little indication of a striking need for chloride by the animal organism.

In experiments by Osborne and Mendel (7) and by St. John (8) there was no decrease in the growth of rats when the chloride content of the diet was reduced to 0.035 and 0.05 per cent respectively. Orent-Keiles, Robinson, and McCollum (5) noted retardation of growth on a low chloride diet of unknown chloride content and Marquis (9) on a diet of 0.01 per cent chloride.

In a paper which appeared when the present work was nearly completed, Voris and Thacker (10) found that a diet containing 0.02 per cent chloride caused a depression of appetite, increased consumption of water, increased heat production, and diminished body gain of nitrogen and energy as well as retarded growth.

The present study was prompted by the knowledge that recent advances in scientific knowledge and technology of the nutritionally essential factors allow the preparation from purified constituents of diets drastically low in chloride, or any other desired component, but apparently adequate otherwise for the biological needs of the rat.

One reason for undertaking the investigation was that from the well known reciprocal relationship between the chloride and bicarbonate concentrations in the blood (11) it can be predicted that a continuous chloride deficiency should produce a chronic state of alkalosis in the animal. Virtually all of the available information on alkalosis has been gained from acute experimental conditions and the study of chronic alkalosis may be expected to yield interesting information on the adaptation of the animal organism to this and its associated state, tetany.

This paper contains the report of the effects of a diet containing 0.012 per

cent chloride or less on the gain in body weight, food and water consumption, and susceptibility to tetany of the rat. Chemical data reported are the changes induced by the deficiency on the chloride, bicarbonate, and pH of the blood and on the excretion of chloride in the urine. Aside from a few chemical analyses of blood and tissue chlorides by Marquis (9) no published data exist on the chemical changes produced in the body by chloride deficiency.

TABLE I
Composition of Basal Diet and Salt Mixtures

Basal diet		Salt mixtures*				
	parts		Controls		Chloride-low diet	
			Diet I	Diet II	With bicarbonate	With nitrate
			gm. per 100 gm. food	gm. per 100 gm. food	gm. per 100 gm. food	gm. per 100 gm. food
Casein	25					
Fat	13†					
Sucrose	55					
Liver extract	0.1‡	Ca ₃ (PO ₄) ₂	1.5			1.5
Cod liver oil	2.5§	CaCO ₃		1.5	1.5	
Supplements per kilo diet		NaCl	1.0			
		NaNO ₃				1.5
		NaHCO ₃		1.0	1.5	
Thiamine hydrochloride	8	KCl	1.75			
Riboflavin	8	K ₂ HPO ₄		1.8	2.0	2.0
Pyridoxine	8	MgSO ₄ ·7H ₂ O	0.6		0.6	0.6
Inositol	8	MgCl ₂ ·6H ₂ O		0.6		
Calcium pantothenate	16	Fe(NH ₄) ₂ (SO ₄) ₂ ·6H ₂ O	0.3	0.3	0.3	0.3
		KI	0.03	0.03	0.03	0.03
		Totals	5.18	5.23	5.93	5.93

* Mn, Cu, Zn, and Co were added to each diet in trace amounts.

† Crisco.

‡ A 50 per cent alcoholic extract of Lilly's liver preparation.

§ Fortified to contain 5000 units of vitamin A per ml.

|| The synthetic water-soluble vitamins were generously supplied by Merck and Company, Inc.

Composition of Diets and Experimental Methods

The composition of the basal diet and salt mixtures used in the experimental work is given in Table I. The salt mixtures of the chief diets, control Diet I and the bicarbonate-containing chloride-low diet, were devised so as to yield the same contents of all the mineral constituents except chloride. The approximate percentages in the two diets were calcium 0.6,

phosphorus 0.55, sodium 0.4, potassium 0.9, and magnesium 0.06. The control diet contained 1.4 per cent chloride and the deficient diet not over 0.012 per cent.¹ Control Diet II and the nitrate-containing chloride-low diets were employed to demonstrate that the bicarbonate and carbonate contents were not responsible for the changes and increases in blood carbon dioxide observed in the chloride-deficient animals.

Blood chloride values were determined on tungstate filtrates of whole blood by the mercuric nitrate titration method of Schales and Schales (12). Urinary chlorides were determined by the same method with the modification that solid benzoic acid was added to maintain the solution automatically at a slightly acid pH. A slightly acid reaction is necessary in order to obtain accurate titrations. The control urines were titrated with a solution of mercuric nitrate about 5 times as concentrated as that specified for blood filtrates, while that of rats on the chloride-low diets was titrated with a solution of the strength specified for blood filtrates.

Urine samples for analysis were obtained from groups of three or four rats kept in a wire metabolism cage placed over a large glass funnel. The feces were separated from the urine by a perforated paraffin plate. At each collection the funnel and plate were washed and the wash water added to the urine.

Carbon dioxide was determined on 1 ml. samples of whole blood collected under oil by the manometric method of Van Slyke and Neill (13). The pH of the blood was determined with a Beckman pH meter, with the special air-tight cell of 0.5 ml. capacity for blood supplied by the Beckman Company. All blood samples for analysis were obtained by heart puncture on rats anesthetized with amytal. Oxalate was used as anticoagulant in all cases except for blood intended for pH estimation, which was maintained fluid with heparin. No restriction was put on the consumption of food or water (distilled).

In one series, the young rats were placed on the experimental diets at the age of 3 weeks; with another series the mothers were first put on these diets 2 days after the young were born. In the latter series, there was no significant difference at 3 weeks of age between the body weights of the young on the control and chloride-low diets. Apparently the weight and general condition of the young rats at weaning depended more on the previous history of the mother than on whether they were fed the control or chloride-deficient diet. For that reason, the results of both series of experiments have been considered together.

¹ Chloride in the chloride-deficient diet was determined by digesting about 100 gm. portions with nitric acid in the presence of AgNO_3 and determining the silver chloride gravimetrically.

RESULTS AND DISCUSSION

Growth and Food and Water Consumption—The statistics for the gain in body weight and the food and water consumption of three groups of rats are given in Table II.

The rats on the chloride-low diets to all outward appearances were normal and on autopsy revealed no apparent deviations from the normal in gross structure. In appearance and behavior they could not be distinguished from the controls. There was no noticeable effect on longevity in the period under observation; namely, 150 days. All the rats on the chloride-low diets survived until they were sacrificed. They gave no evidence of irritability or sluggishness. However, in each group of rats the chloride-de-

TABLE II
Gain in Body Weights and Food and Water Intakes of Rats Maintained on Control and Chloride-Low Diets

	Group I, ♀		Group II, ♂		Group III, ♀	
	Chloride-low, 7 rats	Control, 6 rats	Chloride-low, 2 rats	Control, 2 rats	Chloride-low, 2 rats	Control, 2 rats
	days	days	days	days	days	days
Initial age	21	21	21	21	21	21
Final age.	87	87	87	87	52	52
	gm.	gm.	gm.	gm.	gm.	gm.
Average initial weight	41.1	40.1	38.5	36.5	38.5	36.0
“ final weight	154.7	181.0	236.5	292.5	114.5	136.5
“ gain in weight	113.6	140.9	198.0	256.0	76.0	100.5
“ “ “ “ per day	1.7	2.1	3.0	3.9	2.5	3.2
“ food intake per day	7.9	7.5	10.4	11.2	11.8	9.8
Food intake per gm. gain	4.7	3.6	3.5	2.9	4.7	3.1
Average water intake per day	12.9	16.1	20.7	17.2	13.0	17.0
Water intake per gm. gain in weight	7.6	7.7	6.9	4.4	5.2	5.3

ficient animals gained less weight over a given period of time than did their controls. Though the lower weight was not great enough to be significant in any one group, it was consistent in all groups; so that it may rightly be considered a significant fact from the data of all groups.

Another method used to show the retardation of growth by chloride deficiency was to maintain a group of rats on the chloride-low diet for a time and later change half of the group of rats to the control diet. The results of such an experiment in which seven male rats were maintained on the chloride-low ration for 42 days and then four of the rats were changed to the control diet are plotted in Fig. 1. The change to the control diet caused a striking acceleration in the growth rate of the animals.

The animals on the low chloride diet consumed more food per gm. of gain in body weight than did their controls (Table II). In contrast to the results of Voris and Thacker (10), there was no consistent evidence of a depression of appetite on the part of the chloride-deficient rats. The data of Table II show that the food consumption of the control and deficient animals was not significantly different.

The water consumption per gm. of gain in body weight was the same in the females. The apparently higher water consumption observed for the chloride-deficient males is not conclusive because of the few animals in this group.

Chemical Changes—The electrolyte pattern of the blood is altered in severe chloride deficiency by a decrease in the chloride and an increase in

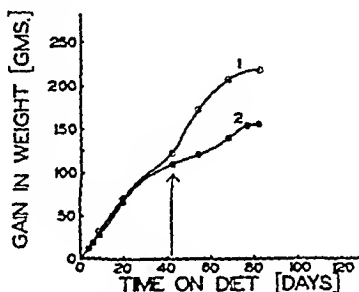


FIG. 1. The acceleration in the growth curve of rats when changed from the chloride-low to the control diet. The time of change is indicated by the arrow. Curve 1, average growth curve of four male rats that were changed to the control diet after 42 days on the chloride-deficient diet. Curve 2, average growth curve of three male rats maintained on the chloride-low diet.

the total carbon dioxide content. The data are summarized in Table III. The blood chloride values were obtained on rats ranging from 55 to 119 days in age, that were on the experimental diets from 32 to 100 days. The body weights ranged from 115 to 300 gm. There was no apparent variation in the chloride values with age, length of time on the diet, or with body weight. The difference between the mean chloride values for the deficient (252 ± 18 mg. per 100 ml.) and the control (295 ± 15 mg. per 100 ml.) animals is definitely significant considering the rather large number of rats tested. The probability of the occurrence of such a difference between the means by chance is less than one in twenty.

The increase in the total carbon dioxide of the blood of the chloride-deficient animals is highly significant, statistically. The probability of the occurrence of the difference in the means between deficient (72.3 ± 4.5

volumes per cent) and control (57.8 ± 4.1) groups is less than one in a hundred.

The greater amount of bicarbonate and carbonate in the diet of the chloride-deficient animals is not responsible for the increase in the carbon dioxide content of the blood. This is demonstrated by the fact that a similar increase in carbon dioxide resulted when the rats were fed the nitrate-containing low chloride ration. No carbonate or bicarbonate whatsoever was present in this diet mixture.

The alkalosis of the chloride-deficient rats is virtually compensated. The difference between the mean pH values of the controls and chloride-deficient rats, 0.06, is statistically insignificant, though there appears to be a tendency toward a higher blood pH in the chloride-deficient animals.

TABLE III

Effect of Chloride Deficiency on Electrolyte Pattern of Blood

The blood chloride figures are measured in mg. of Cl per 100 ml. of blood; the CO₂ in volumes per cent.

Diet	Blood	Control				Chloride-deficient			
		No. of animals	Range	Mean	S.D.	No. of animals	Range	Mean	S.D.
Bicarbon- ate	Cl	42	268 - 338	295	15.4	52	220 - 235	252	18.2
	CO ₂	17	49.8 - 66.5	57.8	4.1	24	60.7 - 79.6	72.3	4.5
	pH	9	7.35 - 7.59	7.46	0.067	11	7.42 - 7.58	7.52	0.053
Nitrate	Cl	3	287 - 314	298		4	207 - 223	216	
	CO ₂	2	58.1 - 59.6	58.9		4	60.7 - 75.2	68.9	

The increase in the carbon dioxide content of the blood does not keep pace with the reduction in chloride concentration. On the basis of the mean values, the increase in mm of carbon dioxide per liter of blood is only one-fourth the decrease that occurs in the chloride concentration.

To study the urinary excretion of chloride, it was found best to determine the change in the rate of excretion of the chloride when rats on the chloride-low diet were changed to the control diet and *vice versa*. Groups of three or four rats were employed to obtain sufficient urine for titration over short time intervals (as low as 1 hour). The chloride-deficient rats excreted 0.02 to 0.05 mg. of halide² per hour per rat, or from 0.5 to 1.2 mg. of halide per day per rat. 5 to 8 hours after they were transferred to the control diet, the excretion of the chloride-deficient animals began to rise and continued to do so for several hours, finally surpassing the usual rate of

² Calculated as chloride.

excretion of the control rats. The excretion dropped back to the normal control level within 24 hours.

The control rats excreted 4.5 to 7.0 mg. of chloride per hour per rat, or 110 to 170 mg. per rat per day. When these rats were changed to the chloride-low ration, the urinary excretion of chloride decreased quickly and within 4 to 11 hours reached the rate of excretion of the rats that had been on the chloride-low diet for a long time. The curves showing the average rates of excretion of chloride by the rats after the change of dietary regimens are plotted in Fig. 2.

The ability of the rat to reduce the excretion of chloride enormously within a few hours explains its ability to withstand a severe deficiency of

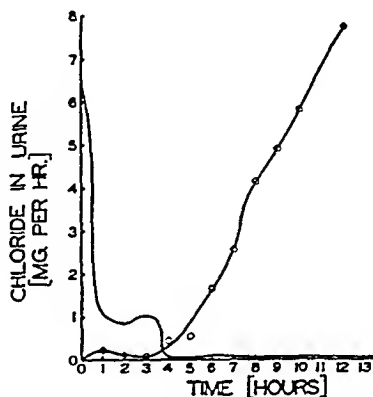


FIG. 2. Excretion of chloride in the urine of rats changed from the chloride-rich to the chloride-low diet (curve on left) and from the chloride-deficient diet to the control diet (curve on right).

this element. There is about a 500-fold difference in the excretion of chloride in the urine between chloride-deficient and control animals. The lowest rate of excretion observed, about 0.5 mg. per rat per day, represents about half the daily chloride intake on the present chloride-low diet. The iodide in the diet could account for 0.6 mg. of halide excretion per day calculated as chloride. Some chloride also must be lost through other excretory channels; *e.g.*, perspiration. It is apparent, however, that the rats are able to retain and accumulate some chloride on a deficient diet which provides an intake of only 1 mg. per day.

A rough calculation from the blood chloride values shows that an adult rat on the chloride-low diet has made a gain of approximately 60 mg. of chloride. Consequently, the degree of chloride deficiency and its accom-

panying chemical and physical changes are due to the increase in the mass of the tissues of the body and not to an actual deficit of body chloride by the animal as a result of excretory loss.

The ability to reduce the urinary excretion to a very low level is also true for sodium (3) and potassium (6). This explains why the intake of these ions and of chloride has to be drastically reduced to produce an actual deficiency.

Tetany—The chloride-deficient rats exhibited no signs of neuromuscular hyperirritability observable by mere inspection, probably because of the nearly completely compensated state of the existing alkalosis. Tests for the existence of tetany have been carried out with the hissing sound of an air blast and a mild galvanic current (see (14)), on a large number of deficient and control rats, usually after they were maintained for 3 to 4 weeks on the experimental rations. A very few of the deficient and none of the control rats went into tetany from the sound of the air blast. A few of the deficient rats exhibited prolonged convulsions of the same tonic epileptiform nature that have been observed in rats with tetany due to thyroparathyroidectomy and to magnesium deficiency (14), when subjected to the galvanic current. However, in the great majority of the cases the galvanic current only caused a fleeting convulsion after prolonged stimulation lasting only for the duration of the current, which could also be produced by the electrical stimulation in similar young control and stock rats.

SUMMARY

Rats were maintained on a chloride-low diet for periods of up to 15 weeks. In spite of the extreme chloride deficiency (0.012 per cent Cl or less in the diet) the rats showed no striking outward signs of deficiency. They gained less weight and ate more food per gm. of gain in body weight than did their controls.

The whole blood chloride values in the chloride-deficient rats were significantly lower than in the controls; the mean for the rats on the chloride-low diet was 252 mg. per 100 ml. and for the controls 295 mg. per 100 ml. The total carbon dioxide content of the blood was significantly higher, the mean being 72.3 volumes per cent as compared to 57.8 volumes per cent for the controls. No significant difference was found in the pH values for whole blood.

The animals were able to conserve their chloride by reducing their urinary output within a few hours after being given the deficient diet. They then excreted 0.5 to 1.2 mg. of Cl per day per rat as compared to a control excretion of 110 to 170 mg. per day per rat.

BIBLIOGRAPHY

1. Heppel, L. A., and Schmidt, C. L. A., *Univ. California Pub. Physiol.*, **14**, 189 (1938).
2. Grijns, G., *Z. physiol. Chem.*, **251**, 97 (1938).
3. Orent-Keiles, E., and McCollum, E. V., *J. Biol. Chem.*, **140**, 337 (1941).
4. St. John, J. L., *J. Biol. Chem.*, **77**, 27 (1928).
5. Orent-Keiles, E., Robinson, A., and McCollum, E. V., *Am. J. Physiol.*, **119**, 651 (1937).
6. Orent-Keiles, E., and McCollum, E. V., *J. Biol. Chem.*, **133**, 75 (1940).
7. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **34**, 131 (1918).
8. St. John, J. L., *J. Agric. Research*, **37**, 55 (1928).
9. Marquis, M., *Compt. rend. Soc. biol.*, **128**, 449 (1938).
10. Voris, L., and Thacker, E. J., *J. Nutrition*, **23**, 365 (1942).
11. Peters, J. P., and Van Slyke, D. D., *Quantitative clinical chemistry*, Interpretations, Baltimore, 1043 (1931).
12. Schales, O., and Schales, S. S., *J. Biol. Chem.*, **140**, 879 (1941).
13. Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).
14. Greenberg, D. M., Boelter, M. D. D., and Knopf, B. W., *Am. J. Physiol.*, in press.

ARTERIAL AND CEREBRAL VENOUS BLOOD CHANGES PRODUCED BY ALTERING ARTERIAL CARBON DIOXIDE*

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Rapid alteration of the carbon dioxide content of the arterial blood in man (1-3) and in anesthetized monkeys (4) modifies both the frequency and amplitude of the electrical potentials obtained from the cerebral cortex. Chemical examination of the internal jugular blood during such procedures (1-3) shows that normally the brain does not passively follow the chemical fluctuations of the arterial blood, because mechanisms are present which tend to prevent alterations of the cerebral carbon dioxide level. The present experiments were designed to test the efficiency of these mechanisms.

Methods

Healthy young men whose electroencephalograms were normal were used as subjects. While lying at rest, the subject breathed from a tube through which a large minute-volume flow of air was driven by a blower. The stream of air was more than sufficient to meet the needs of the subject's maximum pulmonary ventilation. To the air stream, carbon dioxide was added from a pressure tank equipped with a calibrated needle-valve. The carbon dioxide flow was increased in steps and 2 or 3 minutes were allowed between adjustments. A second group of subjects performed vigorous hyperventilation for 6 minutes while lying supine.

In both groups, frequent blood samples were taken, for the most part, simultaneously from indwelling needles in the femoral artery and the internal jugular vein before, during, and after the respiratory procedures. The blood samples were examined chemically for the carbon dioxide content, oxygen content, inorganic phosphorus, and lactic acid by procedures already described (5). The pH of the samples was determined at 38° with the glass electrode, and the carbon dioxide tension and oxygen per cent saturation were calculated from the data.

Results

The data obtained are presented in Tables I and II. These data show that many of the changes in composition of the individual constituents of

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TABLE I

Changes Produced in Arterial and Internal Jugular Blood of Three Subjects by Breathing Air Enriched with Carbon Dioxide

Sample No.	Time	pH at 38°		CO ₂ content		CO ₂ tension		O ₂ content		O ₂ saturation		Serum inorganic P		Lactic acid	
		Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein
	a.m.			vol. per cent	vol. per cent	mm. Hg	mm. Hg	vol. per cent	vol. per cent	per cent	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1V	10.39		7.384		56.3		48.8		11.3		60.7		3.19		16.9
1A	10.44	7.427		50.2		40.2		17.4		93.6		3.19		16.0	
2	10.48	7.451	7.392	50.1	56.2	38.3	47.8	17.5	11.1	93.8	60.0			15.2	16.9
	10.48	Carbon dioxide on													
3	10.51	7.433	7.381	51.0	55.5	40.3	48.4	17.5	11.6	94.0	62.2	3.19	3.19	14.5	16.5
4V	10.56		7.303		59.5		61.1		14.4		77.6				11.0
5	10.57	7.306	7.300	55.9	59.9	57.4	62.0	17.6	16.3	94.4	88.0	3.45	3.40	7.2	9.2
	10.59	Carbon dioxide off													
6	11.00	7.494	7.412	47.2	55.9	33.0	45.5	17.7	9.7	95.1	52.0	3.32	3.33	12.5	15.4
7	11.05	7.413	7.370	50.2	56.0	41.3	50.0	17.6	12.1	95.0	65.6			13.9	15.4
8	11.17	7.433	7.362	50.2	56.0	39.7	50.8	17.4	11.5	93.6	61.8			13.8	15.2
9	11.29	7.430	7.360	50.0	55.5	39.7	50.6	17.5	12.0	93.8	64.5	3.19	3.19	14.5	15.4
1V	10.37		7.384		57.2		51.8		14.6		63.8		2.97		9.5
1A	10.47	7.431		50.3		42.3		21.9		94.0		2.97		8.8	
2	10.49	7.431	7.370	50.3	57.4	42.2	53.5	21.9	14.8	94.0	64.5			9.0	9.5
	10.54	Carbon dioxide on													
3	10.56	7.431	7.391	50.1	56.8	42.1	51.1	22.0	15.4	94.6	67.5	2.99	2.97	11.6	12.6
4	10.58	7.289	7.207	59.6	59.5	66.2	64.4	22.1	18.5	95.0	81.0	2.99	3.01	9.3	10.6
	10.59	Carbon dioxide off													
5	11.00	7.439	7.378	49.5	54.3	40.8	49.4	22.0	16.7	94.2	73.0	2.97	2.97	6.0	7.2
6	11.06	7.470	7.373	48.8	56.4	37.9	52.2	22.0	14.8	94.2	64.9			7.5	9.7
7	11.17	7.421	7.371	50.4	56.5	43.0	52.5	21.9	15.0	93.8	65.4	3.09	3.09	11.5	12.1
8	11.28	7.425	7.366	50.3	57.4	42.8	53.7	21.8	14.4	93.6	62.8	3.09	3.09	10.2	10.4
1V	10.11		7.373		54.5		50.2		14.0		64.8		2.93		15.5
1A	10.15	7.434		48.3		39.5		20.5		93.9		2.93		12.5	
2	10.17	7.423	7.375	48.4	54.2	40.8	49.5	20.5	14.3	93.8	65.7			11.5	13.8
	10.23	Carbon dioxide on													
3	10.24	7.443	7.378	47.5	54.5	38.2	49.3	20.7	13.9	94.8	63.5	2.98	3.04	9.1	10.5
4	10.27	7.368	7.347	51.1	54.7	47.9	52.8	20.6	16.6	94.6	75.6	2.98	3.04	8.7	9.6
5	10.29	7.348	7.310	52.6	56.0	51.2	58.7	20.6	17.9	94.6	82.6	3.03	3.04	8.2	9.6
6	10.31	7.316	7.286	53.8	56.8	55.7	62.7	20.8	19.2	95.2	88.6	3.04	3.04	10.0	10.8
	10.32	Carbon dioxide off													
7	10.33	7.430	7.368	45.5	52.6	37.5	48.9	20.8	15.0	95.0	68.5	3.04	3.04	14.5	16.1
8	10.38	7.428	7.370	47.0	53.3	39.0	49.1	20.6	14.6	94.6	66.8	3.04	3.04	15.4	15.9
9	10.48	7.426	7.371	48.7	54.6	40.5	50.0	20.5	13.6	93.7	62.4	3.04	3.01	11.5	13.3
10	11.02	7.413	7.368	48.2	54.2	41.0	50.2	20.6	14.5	94.0	66.3	3.04	3.04	9.9	10.9

the arterial and internal jugular blood can be correlated with the primary effect, the alteration of the level of carbon dioxide in the arterial blood. Slight but definite alterations occur in the concentration of inorganic phos-

TABLE II

Changes Produced in Arterial and Internal Jugular Blood of Three Subjects by Voluntary Hyperventilation

Sample No.	Time	pH at 38°		CO ₂ content		CO ₂ tension		O ₂ content		O ₂ saturation		Serum inorganic P		Lactic acid	
		Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein	Artery	Vein
	a.m.			vol. per cent	vol. per cent	mm. Hg	mm. Hg	vol. per cent	vol. per cent	per cent	per cent	mg. per cent	mg. per cent	mg. per cent	mg. per cent
IV	9.54		7.335		56.6		54.8		11.9		61.1		3.23		11.2
2	9.59	7.374	7.321	49.8	56.5	44.9	56.3	18.4	12.0	93.5	61.6	3.18	3.23	8.8	11.8
	10.02	Overventilation begun													
3	10.04	7.583	7.420	42.7	56.7	25.2	45.8	18.7	8.5	95.0	43.6	3.03	3.01	8.4	10.4
4	10.05	7.551	7.442	40.4	55.5	25.4	42.7	18.9	7.4	96.0	38.0	2.96	2.99	9.6	15.5
5	10.07	7.622	7.479	38.4	53.4	20.9	38.1	18.8	6.3	95.5	32.3	2.90	2.91	11.3	16.8
	10.07	Definite signs of tetany													
	10.08	Overventilation over													
6	10.10	7.544	7.508	46.3	52.3	29.6	35.3	18.0	9.3	91.5	47.7	3.00	3.00	13.3	15.4
7	10.19	7.419	7.345	48.3	53.1	39.9	50.2	18.5	11.9	94.0	61.1	3.01	3.02	10.5	12.1
8V	10.35		7.363		55.5		50.7		11.6		59.5				16.6
9	10.39	7.390	7.345	49.3	56.6	43.0	53.5	18.4	11.8	93.5	60.5	3.00	3.00	14.0	15.3
IV	10.40		7.375		53.1		47.2		11.4		58.6		2.66		10.9
1A	10.43	7.440		46.6		36.6		18.2		93.6		2.66		9.0	
2	10.49	7.438	7.375	46.4	53.2	36.7	47.3	18.1	11.2	93.1	57.6			8.5	10.7
	10.52	Overventilation begun													
3	10.53	7.572	7.454	38.7	53.3	23.3	40.0	18.7	8.9	96.1	45.8	2.57	2.57	10.8	14.2
4	10.55	7.611	7.474	36.6	51.8	20.3	36.9	18.6	7.6	95.6	39.1	2.48	2.48	11.0	13.8
5	10.58	7.667	7.524	35.8	49.5	17.7	32.1	18.7	7.1	96.1	36.5	2.40	2.43	15.1	16.1
	10.58	Overventilation over													
6	11.00	7.535	7.464	40.8	51.7	26.3	38.1	17.5	8.9	90.0	45.8	2.37	2.42	13.2	16.2
7	11.06	7.444	7.409	44.8	51.9	34.7	42.8	15.2	9.4	78.1	48.4	2.21	2.26	15.2	17.9
8V	11.16		7.393		52.8		45.2		11.8		60.7		2.21		
9	11.21	7.425	7.363	46.1	53.1	37.4	48.3	18.1	11.4	93.1	58.6	2.18	2.19	14.8	15.9
IV	11.11		7.396		52.9		47.1		15.3		64.4		3.50		11.9
1A	11.13	7.448		46.0		37.4		22.3		93.6		3.48		10.6	
2	11.17	7.446	7.386	45.8	52.2	37.6	47.7	22.2	16.6	93.5	60.9			10.1	11.3
	11.18	Overventilation begun													
3	11.19	7.551	7.444	39.3	52.4	26.0	42.1	22.5	12.8	94.5	54.0		3.42	11.8	12.6
4	11.21	7.591	7.455	36.8	51.2	22.5	40.2	22.6	10.7	94.9	44.7		3.35	12.5	14.1
5	11.23	7.633	7.499	34.7	49.7	19.5	35.6	22.8	9.7	95.8	40.5	3.05	3.07	13.3	14.4
	11.24	Overventilation over													
6	11.25	7.520	7.461	43.3	49.9	30.3	38.7	20.4	11.9	86.2	50.0	2.99	3.03	12.5	13.6
7	11.29	7.474	7.419	44.6	50.2	32.9	42.7	20.0	13.8	84.0	58.1			11.5	11.3
8	11.35	7.441	7.412	45.6	50.4	37.8	43.4	21.7	14.1	91.2	59.1	2.99	3.03	8.4	11.1
9	11.44	7.444	7.376	46.2	51.4	38.0	47.6	22.2	14.6	93.5	61.2			9.2	10.8
10	11.54	7.441	7.373		52.8		49.1		15.0		63.3		2.91	10.6	11.9

phorus which, though not parallel to the changes in carbon dioxide, are in the same direction. Also, a definite inverse relationship is apparent be-

tween the lactic acid and the carbon dioxide levels. When carbon dioxide-enriched air is breathed, a decrease in lactic acid occurs, in spite of the violent activity of the respiratory muscles. The short period effects of carbon dioxide on lactic acid that appear here are entirely in accord with the earlier results of Anrep and Cannan (6) on the heart-lung preparation, and of Long, (7) on man, when the carbon dioxide levels were altered for longer

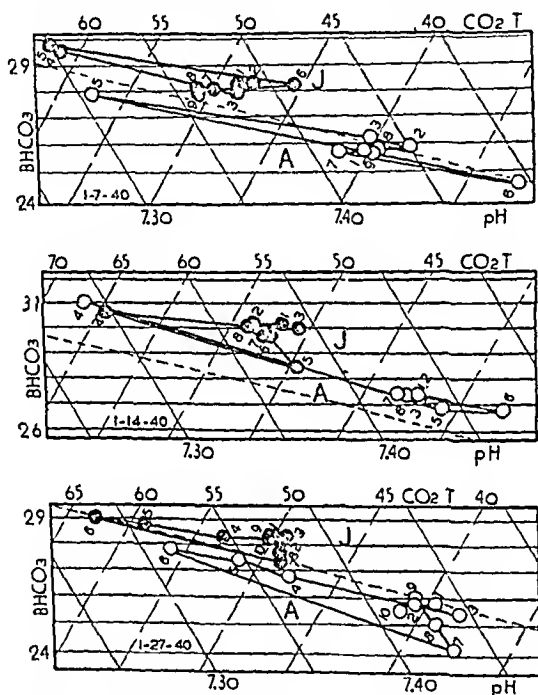


FIG. 1. The acid-base displacement and recovery paths produced in the three individuals shown in Table I, breathing carbon dioxide-enriched air for short periods. O, arterial blood; \odot , internal jugular blood; BHCO_3 , mm. per liter of serum; $\text{CO}_2 \text{ T}$, carbon dioxide tension in mm. of mercury. The figures placed beside the dots indicate the order in which blood samples were drawn.

periods of time. The present results emphasize the speed with which such changes are brought about.

Comparison of the fluctuations of pH, carbon dioxide tension, and carbon dioxide content of the arterial and internal jugular blood reveals that the brain is protected from rapid alteration of its acid-base balance, for the quantitative changes in the arterial blood are much greater than in the venous blood. The degree of protection is somewhat greater against a decreased level of arterial carbon dioxide than against an increased level.

Figs. 1 and 2 show the acid-base displacement paths (8), for increased and decreased levels of arterial carbon dioxide respectively, and they show the

relationship that exists between simultaneously drawn samples of arterial and internal jugular blood. From Fig. 1 it may be seen that when carbon dioxide-enriched air is breathed the arterial and the cerebral venous bloods become more nearly alike as regards their acid-base characteristics. In

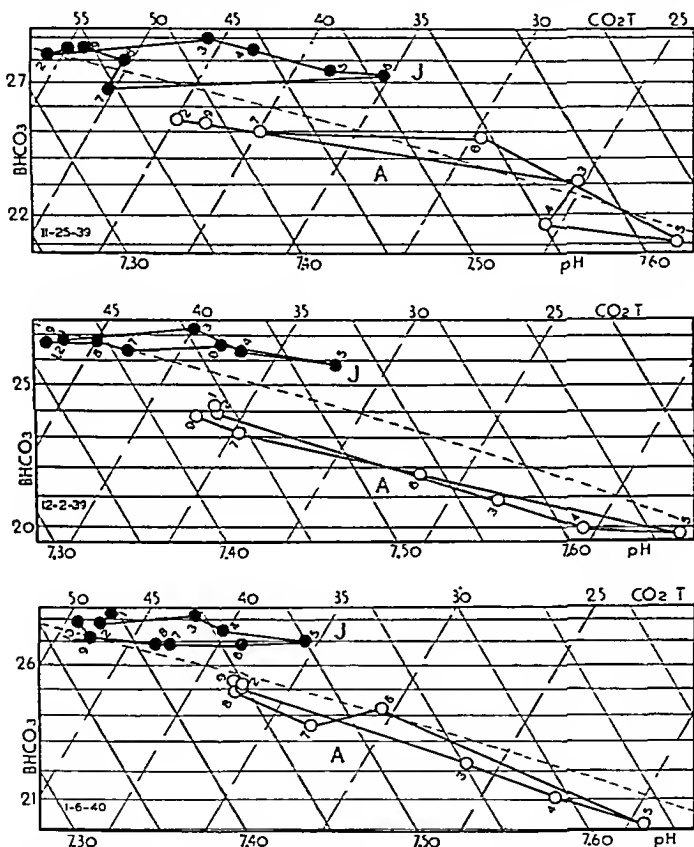


FIG. 2. The acid-base displacement and recovery paths produced in the three individuals shown in Table II by vigorous hyperventilation. Symbols and abbreviations as in Fig. 1. Note the difference in slope between the path for the internal jugular blood and the arterial blood.

contrast, as shown in Fig. 2, when the lungs are overventilated, the difference between arterial and cerebral venous blood is accentuated.

The dash line through the center of these plots is an *in vitro* titration curve of blood with carbon dioxide (9). It should be noticed how closely

the *in vivo* titration curve of arterial blood with carbon dioxide parallels the slope of the *in vitro* curve. The slope of the internal jugular blood displacement path, particularly during hyperventilation, is quite different. This is due to the fact that only a very small change is produced in the base bicarbonate of the cerebral venous blood by hyperventilation.

In the normal individuals studied here, the displacement paths are retraced quite closely during recovery. This behavior is distinctly different from that observed in the petit mal epileptics previously studied (10). In

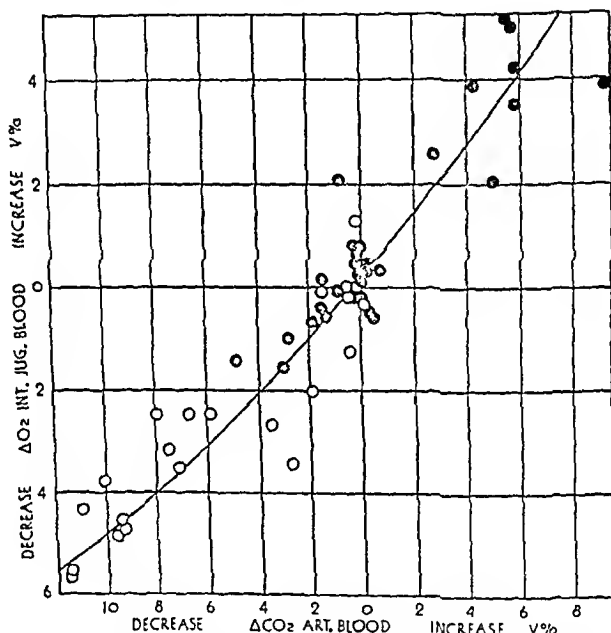


FIG. 3. The relation between the change in carbon dioxide level of the arterial blood and the change in oxygen level in the internal jugular blood; data from eight individuals. O, experiments in which hyperventilation was performed; ●, experiments in which carbon dioxide-enriched air was breathed.

these patients the displacement path during hyperventilation is not retraced during recovery, with the result that loops are produced.

Although the circulation of the brain does protect the brain somewhat from arterial changes in acid-base balance, it does so only at the expense of wide fluctuations in oxygen level. The observed saturation of oxygen in the internal jugular blood went as high as 88 per cent during inhalation of carbon dioxide mixtures, and as low as 32 per cent during hyperventilation. Fig. 3 indicates a close relationship between the arterial carbon dioxide level and the internal jugular oxygen level. A change of 1 volume per cent from the preliminary value in the carbon dioxide level of the arterial blood pro-

duces a change of 0.5 volume per cent in the oxygen level of the cerebral venous blood. From this it is clear that a sudden increase in arterial carbon dioxide causes an increased oxygen supply to the brain.

DISCUSSION

The chemical findings presented here confirm the evidence collected by Lennox and his coworkers (1-3) that at normal oxygen pressures the brain is protected from rapid changes in its acid-base balance. This protection is largely achieved by an alteration of blood flow; the diameter of the blood vessels closely follows the acid-base balance of the arterial blood, as the data presented in Fig. 3 demonstrate. The limits through which the regulation of blood flow serves to stabilize the acid-base balance of the brain are only slightly greater than are covered by the present data; as has already been demonstrated (2), the constriction of the cerebral vessels to an alkaline state of the arterial blood is released if the oxygen saturation of the internal jugular blood falls below 30 per cent. Under such conditions, the mechanism protecting the acid-base equilibrium of the brain is sacrificed by as yet unknown means to the need of the brain for oxygen.

SUMMARY

Chemical examination of the internal jugular blood of healthy young men, when rapid increases or decreases of carbon dioxide in the arterial blood are produced, reveals the presence of effective mechanisms which serve to protect the brain from alterations of its acid-base balance.

A direct relation between the inorganic phosphorus and carbon dioxide levels of the blood and an inverse relation between the lactic acid and carbon dioxide levels have been found during changes in carbon dioxide concentration of the arterial blood when these changes are produced by breathing carbon dioxide-enriched air and by pulmonary overventilation.

BIBLIOGRAPHY

1. Lennox, W. G., and Gibbs, E. L., *J. Clin. Inv.*, **11**, 1155 (1932).
2. Lennox, W. G., Gibbs, F. A., and Gibbs, E. L., *J. Neurol. and Psychiat.*, **1**, 211 (1938).
3. Gibbs, E. L., Gibbs, F. A., Lennox, W. G., and Nims, L. F., *Arch. Neurol. and Psychiat.*, **47**, 879 (1942).
4. Dusser de Barenne, J. G., McCulloch, W. S., and Nims, L. F., *J. Cell. and Comp. Physiol.*, **10**, 227 (1937).
5. Gibbs, E. L., Lennox, W. G., Nims, L. F., and Gibbs, F. A., *J. Biol. Chem.*, **144**, 325 (1942).
6. Anrep, G. V., and Cannan, R. K., *J. Physiol.*, **58**, 244 (1923).
7. Long, C. N. H., *J. Physiol.*, **58**, 455 (1924).
8. Sbock, N. W., and Hastings, A. B., *J. Biol. Chem.*, **112**, 239 (1935-36).
9. Henderson, L. J., *Blood, A study in general physiology*, New Haven (1928).
10. Nims, L. F., Gibbs, E. L., Lennox, W. G., Gibbs, F. A., and Williams, D., *Arch. Neurol. and Psychiat.*, **43**, 262 (1940).

THE EFFECT OF SOME INORGANIC PLANT NUTRIENTS ON MALT DIASTASE ACTIVITY

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(Received for publication, June 25, 1942)

It has long been known that the activity of diastase may be altered by the addition of salts to the medium. Increase in the activity of diastase can be brought about by the addition of sodium fluoride, sodium chloride, calcium chloride, potassium chloride, barium chloride, and magnesium chloride, according to Haehn and Schweigart (1). They also reported a decrease in activity of diastase by the addition of zinc, cadmium, lead, and copper. It may also be noted that they did not observe complete inactivation of the enzyme in the absence of a salt. Haehn and Schweigart (1) cite W. S. Iljin as stating that the concentration of the salt determines whether enzyme activity is increased or depressed. An increase in diastase activity was observed by Sherman and Thomas (2) when they added sodium chloride, potassium chloride, sodium nitrate, sodium sulfate, primary sodium phosphate, and primary potassium phosphate to their solutions. James and Cattle (3) concluded that potassium chloride increased diastase activity of leaves and that the chloride ion activates the diastase strongly, while the potassium ion increases the amount of diastase formed in leaves. Doby and Hibbard (4) state that diastase is strongly activated by the chloride ion and weakly by the potassium, nitrate, and fluoride ion. In this study it was desired to ascertain the effect of some plant nutrient elements on malt diastase activity.

EXPERIMENTAL

A commercial potato starch, purified by dialyzing in a collodion bag against distilled water for 5 days, was used for the substrate. A commercial malt diastase preparation was purified in the same manner. The colorimetric method was used for starch determination and was essentially the same method as that described by Paloheimo (5), Paloheimo and Paloheimo (6), and Paloheimo and Antila (7). Light intensity was measured by use of a photometer equipped with a Cenco orange filter No. 4. The medium was not buffered, for it is not known what effect a buffer has on enzyme activity.

The compounds considered in this study, manganese chloride, zinc chloride, iron chloride, copper chloride, and boric acid, are essential for plant growth in relatively very small amounts. Comparisons were made

on the basis of ionic strength, except for boric acid. Fig. 1 shows the effect of the four salts on diastase activity when the media had a salt concentration of 0.0003 ionic strength. Manganese chloride and zinc chloride increased diastase activity, while copper chloride and iron chloride decreased diastase activity, as compared to a medium containing no salt compound. The order of activation is not the same throughout the experiment. Copper chloride depressed diastase activity less than iron chloride to a starch concentration of 0.0056 per cent, after which copper chloride depressed diastase activity more than iron chloride. When the media had a salt concentration of 0.003 ionic strength, the rate of diastase activity was depressed, except with manganese chloride, which increased diastase activity up to an ionic strength of 0.3. However, when the solutions

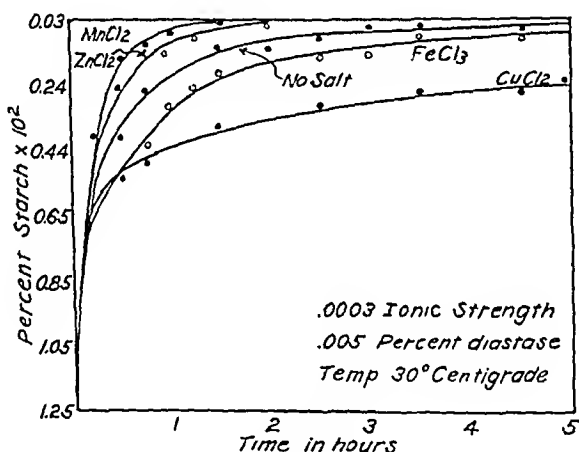


FIG. 1. Effect of the four salts on malt diastase activity

had an ionic strength of 0.00003, the rate of diastase activity was approximately the same as when no salts were added. When the four salts were mixed in the same medium and the solution had a total ionic strength of 0.0003, the rate of diastase activity was also approximately the same as when no salts were added.

The results seem to indicate that the metallic ion influences diastase activity strongly, while the chloride ion has little effect on diastase activity, for the effect of an increase or decrease in concentration of a salt is correlated with the metallic ion and not with the chloride ion.

In comparing the effect of boric acid on diastase activity we found that in a 0.00001 molal solution approximately the same activity rate was obtained as when no salt compound was present, but in a 0.0001 molal solution or stronger the activity was depressed.

SUMMARY

It was found that the effect on diastase activity of the compounds considered in this study depends not only on the kind of compound but also on the concentration of the compound in the medium. Under the conditions of the experiment manganese chloride increased diastase activity up to an ionic strength of 0.3, zinc chloride up to an ionic strength of 0.0003, while iron chloride, copper chloride, and boric acid did not increase diastase activity in any concentration employed.

BIBLIOGRAPHY

1. Haehn, H., and Schweigart, H., *Biochem. Z.*, **143**, 516 (1923).
2. Sherman, H. C. and Thomas, A. W., *J. Am. Chem. Soc.*, **37**, 623 (1915).
3. James, W. O., and Cattle, M., *New Phytologist*, **34**, 233 (1935).
4. Doby, G., and Hibbard, R. P., *J. Biol. Chem.*, **73**, 405 (1927).
5. Paloheimo, L., *Biochem. Z.*, **222**, 150 (1930).
6. Paloheimo, L., and Paloheimo, I., *Biochem. Z.*, **238**, 391 (1931).
7. Paloheimo, L., and Antila, I., *Biochem. Z.*, **238**, 401 (1931).

THE REPLACEABILITY OF *dl*-METHIONINE IN THE DIET OF THE RAT WITH ITS α -KETO ACID ANALOGUE*

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(Received for publication, June 22, 1942)

There is strong evidence for the belief that methionine may be oxidatively deaminated in the animal organism. Not only has it been shown that α -keto- γ -methiolbutyric acid is formed by the action of kidney slices on methionine under proper conditions (2), but also that rats fed large amounts of this sulfur-containing amino acid excrete the corresponding α -ketonic acid in the urine (3). Although a number of compounds closely allied to methionine, including the corresponding sulfoxide (4), sulfone (5), and methylsulfonium chloride (5), as well as homocystine (6) and α -hydroxy- γ -methiolbutyric acid (7), have been studied with reference to their effectiveness in replacing methionine for growth purposes in the diet of young rats, it has remained for the α -keto acid analogue of the latter amino acid to be investigated in this regard.

The preparation of α -keto- γ -methiolbutyric acid for the present study involved splitting the corresponding 2,4-dinitrophenylhydrazine derivative by heating under pressure with aqueous acetone, a procedure similar to that used successfully by Collatz and Neuberg (8) to prepare crystalline dihydroxyacetone from its hydrazone. In the present instance the appropriate hydrazone was prepared in quantity by taking advantage of a reaction reported some years ago by Bergmann and Stern (9). These investigators observed that the chemical oxidative deamination of alanine could be effected by converting the amino acid to its α -bromopropionyl derivative, treating the latter with acetic anhydride and sodium acetate to form a halogen-free azlactone, and subsequently heating the mixture with dilute acid. An intramolecular oxidation-reduction reaction is involved in which the α -carbon of the amino acid is oxidized to a keto group, and the halogenated fatty acid is reduced to the non-substituted fatty acid. We have found that methionine can be oxidatively deaminated in a similar manner.

* A preliminary report was presented before the Thirty-sixth annual meeting of the American Society of Biological Chemists at Boston, 1942 (1).

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The data in this paper are taken from a thesis submitted by Guilford G. Rudolph in partial fulfilment of the requirements for the degree of Master of Science in the Graduate School of Wayne University.

EXPERIMENTAL

Preparation of α -Bromopropionylmethionine—3 gm. of *dl*-methionine (Hoffmann-La Roche) were dissolved in 20.1 cc. of *N* NaOH and the solution was placed in an ice bath. At the end of each 5 minute interval during the course of 1 hour, 1.7 cc. of NaOH and 0.36 gm. of α -bromopropionyl bromide were added to the cold solution, which was agitated vigorously after each addition to facilitate the reaction. After the final addition of alkali and bromide, the solution was allowed to stand for $\frac{1}{2}$ hour at room temperature and was finally filtered into an evaporating dish and acidified with 3.4 cc. of 6 *N* HCl. The solution was then concentrated to about one-fourth of its volume by allowing it to stand several hours in an evacuated desiccator over anhydrous calcium chloride and phosphoric anhydride. The colorless prismatic crystals which formed were filtered off and, after being washed with cold water and thoroughly dried, they weighed 3.16 gm. The *N* calculated is 4.93 per cent; found, 4.79. After recrystallization from water, the compound melted at 111.5–112.5° (corrected) and contained 4.91 per cent *N*. For preparation of the hydrazone the unrecrystallized material was used.

Preparation of the 2,4-Dinitrophenylhydrazone of α -Keto- γ -methiolbutyric Acid—3 gm. of α -bromopropionylmethionine were stirred for 10 minutes at room temperature in a large Pyrex test-tube with 1.7 gm. of freshly fused sodium acetate and 9.5 cc. of acetic anhydride. 24 cc. of *N* HCl were added and the mixture was heated in a boiling water bath for 5 minutes and subsequently cooled. The contents of the tube were then mixed with 125 cc. of 2 *N* HCl which contained 2.1 gm. of 2,4-dinitrophenylhydrazine. The hydrazone which formed immediately was filtered off after the flask had stood overnight in the refrigerator. After recrystallization from dilute methanol and from water, the hydrazone was further purified by dissolving it in a 2 per cent sodium carbonate solution and precipitating with HCl. After again being crystallized from water, 1.3 gm. of yellow micro crystalline hydrazone were obtained which melted at 148–149°. The *N* calculated is 17.07 per cent; found, 17.04. The nitrogen was determined by the modified Kjeldahl method of Šimek (10), which involves a preliminary reduction with sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) and which is designed for this type of compound. Waelsh and Borek (2) have reported a melting point of 149° for the compound which was prepared by allowing kidney slices to deaminate methionine oxidatively, and then precipitating the keto acid as the 2,4-dinitrophenylhydrazone.

Splitting the 2,4-Dinitrophenylhydrazone—2 gm. of the 2,4-dinitrophenylhydrazone of α -keto- γ -methiolbutyric acid were dissolved in 120 cc. of a 50 per cent aqueous solution of acetone and the mixture was heated for 4 hours in a 200 cc. pressure bottle which was kept in a constant temperature

water bath at 90–92°. The flask was then kept in the refrigerator overnight, after which the contents were concentrated to about 40 cc. *in vacuo*. The unsplit hydrazone and the 2,4-dinitrophenylhydrazone of acetone were filtered off, and, after neutralization with 0.5 N barium hydroxide, the filtrate was taken to dryness in the vacuum desiccator over P_2O_5 and $CaCl_2$. Several 2 gm. samples of the hydrazone were treated in this manner. In each case the unsplit hydrazone was separated from the 2,4-dinitrophenylhydrazone of acetone by dissolving in 2 per cent sodium carbonate solution, filtering, and reprecipitating with HCl. The precipi-

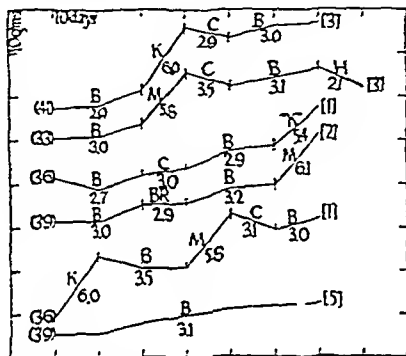


Fig. 1. Growth of rats fed a basal arachin diet and a supplemented basal diet. The letters indicate the diets employed during the experimental period: Diet B, basal arachin diet; Diet K, 513 mg. of sodium α -keto- γ -methiolbutyrate per 100 gm. of basal diet; Diet M, 450 mg. of *dl*-methionine per 100 gm. of basal diet; Diet C, 360 mg. of *l*-cystine per 100 gm. of basal diet; Diet "K," 31 mg. of sodium α -keto- γ -methiolbutyrate in water injected subcutaneously each day in a rat fed the basal arachin diet; Diet H, 982 mg. of 2,4-dinitrophenylhydrazone of α -keto- γ -methiolbutyric acid per 100 gm. of basal diet; Diet BR, 858 mg. of α -bromopropionyl-*dl*-methionine per 100 gm. of basal diet. The figures in parentheses indicate the initial weights of the animals; those in brackets, the number of animals represented by the curve. The figures below the curves indicate the average daily food consumption in gm. for each interval.

tate was then again subjected to the acetone treatment. From 9.5 gm. of the hydrazone 3.0 gm. of the barium salt of α -keto- γ -methiolbutyric acid were obtained, which after decolorization with activated charcoal contained 32.55 per cent Ba and 15.41 per cent S. (Calculated, Ba 31.82, S 14.85.) The salt did not give a test for nitrogen and gave a precipitate with 2,4-dinitrophenylhydrazine which dissolved in a sodium carbonate solution with the production of the deep red color that is characteristic of the 2,4-dinitrophenylhydrazone of α -keto acids treated in this manner. The sodium salt of the keto acid was used in the animal experiments. It

was prepared by treating an aqueous solution of the barium salt with the requisite amount of Na_2SO_4 and centrifuging to remove the BaSO_4 .

Animal Experiments—Sprague-Dawley rats were fed a methionine-low diet which was identical with that used by White and Beach (6), except that starch was replaced with dextrin. Arachin for the diet was prepared from peanut meal by the method of Johns and Jones (11). The basal diet was supplemented with the sodium salt of α -keto- γ -methiolbutyric acid, or with *dl*-methionine, or with other compounds as indicated in Fig. 1. As may be seen in Fig. 1, the keto acid derived from methionine was, like *dl*-methionine itself, effective in promoting the growth of rats fed a diet low in this amino acid, whereas neither α -bromopropionyl-*dl*-methionine nor the 2,4-dinitrophenylhydrazine derivative of the keto acid was effective in this respect. Cystine, included for comparison, also had no comparable effect, as has been previously shown (6). The average daily gain of the rats during the period in which they were fed the diet supplemented with the keto acid was 1.3 gm., while the rats provided with *dl*-methionine gained an average of 1.25 gm. daily. The food consumption of the rats was also increased when the diet was supplemented with methionine or its keto acid analogue, as shown in Fig. 1. The subcutaneous injection of an aqueous solution of the sodium salt of the keto acid in a rat fed the methionine-low diet also promoted the growth of the animal as is likewise indicated. Owing probably to leakage of some of the injected keto acid solution, however, the parenteral route was not as effective as the oral in promoting growth in this instance.

Jackson and Block (12) have pointed out that the utilization of *d*-methionine as well as of the *dl*- α -hydroxy and the *dl*-N-methyl derivatives of this amino acid "... seems to speak for processes involving a common metabolic intermediate and, in particular, the corresponding α -ketonic acid." Indeed, on the basis of indirect evidence obtained in a study of N-methyl-methionine, Patterson, Dyer, and du Vigneaud (13) have inferred that the keto acid derived from methionine would be utilizable in place of the amino acid. The present experiment bears out this prediction.

SUMMARY

1. The preparation of a salt of α -keto- γ -methiolbutyric acid by a procedure involving the chemical oxidative deamination of methionine is described.
2. The sodium salt of the α -keto acid derived from methionine is capable of replacing *dl*-methionine for growth purposes in young rats.

BIBLIOGRAPHY

1. Cahill, W. M., and Rudolph, G. G., *Federation Proc.*, **1**, pt. 2, 104 (1942).
2. Waelsch, H., and Borek, E., *J. Am. Chem. Soc.*, **61**, 2252 (1939).

3. Waelsch, H., *J. Biol. Chem.*, **140**, 313 (1941).
4. Bennett, M. A., *Biochem. J.*, **33**, 1794 (1939).
5. Bennett, M. A., *J. Biol. Chem.*, **141**, 573 (1941).
6. White, A., and Beach, E. F., *J. Biol. Chem.*, **122**, 219 (1937-38). Rose, W. C., and Rice, E. E., *J. Biol. Chem.*, **130**, 305 (1939). du Vigneaud, V., Dyer, H. M., and Kies, M. W., *J. Biol. Chem.*, **130**, 325 (1939).
7. Block, R. J., and Jackson, R. W., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **97**, p. evi (1932). Akobe, K., *Z. physiol. Chem.*, **244**, 14 (1936).
8. Collatz, H., and Neuberg, I. S., *Biochem. Z.*, **255**, 27 (1932).
9. Bergmann, M., and Stern, F., *Ann. Chem.*, **448**, 20 (1926).
10. Šimek, B. G., *Chem. Listy*, **25**, 322 (1931); *Chem. Abst.*, **25**, 5871 (1931).
11. Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, **28**, 77 (1916-17).
12. Jackson, R. W., and Block, R. J., *J. Biol. Chem.*, **122**, 425 (1937-38).
13. Patterson, W. I., Dyer, H. M., and du Vigneaud, V., *J. Biol. Chem.*, **116**, 277 (1936).

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CHOLINE AND THE PREVENTION OF HEMORRHAGIC KIDNEYS IN THE RAT

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Three functions have been ascribed to choline in animal metabolism: (a) the prevention of fatty livers, due according to Welch (1) to the formation of phospholipids and consequent removal of neutral fat from the liver; (b) the prevention of a depressed function of the vagus nerve, which accompanies a dietary deficiency of choline, ascribed by Solandt and Best (2) to an inadequate formation of acetylcholine; (c) the supply of labile methyl groups, for example, for the methylation of homocystine, reported by du Vigneaud and associates (3).

Griffith and Wade (4) have described the production of renal hemorrhage in very young rats maintained on a low choline diet; Griffith and Mulford (5) postulated that the renal degeneration was due to a lack of labile methyl groups, mainly because the feeding of methionine would prevent the lesion. Welch (6) observed that renal hemorrhage could be prevented by supplying triethylcholine, a substance which has been shown not to furnish methyl groups (3). Because of this observation, and because of the evidence regarding the relation of lipotropic action to phospholipid formation, it seemed possible that the renal damage might be due to a failure of phospholipid formation in the liver with consequent phospholipid deficiency in the kidney. Griffith and Wade (4) have stated that renal hemorrhage can be produced most easily in only one brief portion of the rat's life, that immediately after weaning. This is a period of rapid growth, during which a supply of phospholipid may be particularly essential for the production of normal cellular structure.

The observations reported in this paper deal with the possible correlation between alterations in the phospholipid content of kidneys and the coincident production of renal hemorrhage.

Method

Young male albino rats of the Wistar strain reared in the Connaught Laboratories colony were used at an initial weight of 40 to 45 gm., a range corresponding to that used by Griffith and associates (4). The animals were housed in individual screen bottom cages, with water supplied *ad libitum*. The basal, low choline diet had the following composition: casein (Labco, vitamin-free), 10.0 per cent; corn oil (Mazola), 30.0; sucrose

(Redpath), 53.5; salt mixture (Steenbock-Nelson, Salts 40 (7)), 4.0; agar (British Drug Houses), 2.0; *l*-cystine (Pfanstiehl), 0.5.

To every 100 gm. of diet, 0.015 gm. of cod liver oil concentrate (Ayerst, McKenna and Harrison) was added to supply vitamins A and D. Each animal received a daily supplement, given by injection, containing 20 γ of thiamine chloride, 20 γ of riboflavin, 100 γ of calcium pantothenate, and 20 γ of pyridoxine.

All animals were killed by stunning after 10 days, except in the case of Series V. The liver and kidneys were removed and phospholipid determinations were made by the method of Bloor (8). Each group in Series I to IV contained ten animals and all results are given as arithmetical averages for each group.

TABLE I
Phospholipid Content of Rat Kidneys

Series No.	Lipotropic supplement	Animals with renal hemorrhage	Average kidney weight	Average kidney phospholipid concentration	Phospholipid, total content
		<i>per cent</i>	<i>gm.</i>	<i>per cent</i>	<i>mg.</i>
I	None	60	0.80	1.9	15.5
	20 mg. choline per day, injected	0	0.67	2.7	18.2
II	None	90	1.15	1.6	18.4
	20 mg. choline per gm. food	0	0.78	2.5	19.6
III	None	90	1.11	1.5	16.3
	3 mg. triethylcholine per gm. food	0	0.74	2.3	16.8
IV	None	80	0.98	1.7	16.8
	5 mg. triethylcholine per gm. food	0	0.62	2.4	14.6

EXPERIMENTAL

Two groups of rats were used in each of Series I to IV, one group receiving no lipotropic supplement, the other being given choline or triethylcholine as indicated in Table I. The results are shown in this table.

Series V was designed to show the rate of development of hemorrhagic kidneys and coincident changes of the kidney and liver phospholipids. The series was begun with 55 rats, five of these being killed on the 1st day. The rest were divided into two groups, one of which received 2 mg. of choline per gm. of food, while the other was given no choline. Five animals of each group were killed on the 2nd, 4th, 6th, 8th, and 10th days. Changes in the average weight of the kidneys and in the average concentration of phospholipids in the kidneys are given in Fig. 1.

On the 10th experimental day all choline-deficient rats in all series showed loss of body weight, paralysis of the hind limbs, loss of hair, and a hunched

position. On autopsy the kidneys of most of these animals (percentages are given in Table I) were much enlarged and hemorrhagic. The livers showed typical fatty infiltration and were slightly larger than those of the animals receiving choline. In each case the administration of a lipotropic supplement completely prevented the macroscopic picture of kidney lesions.

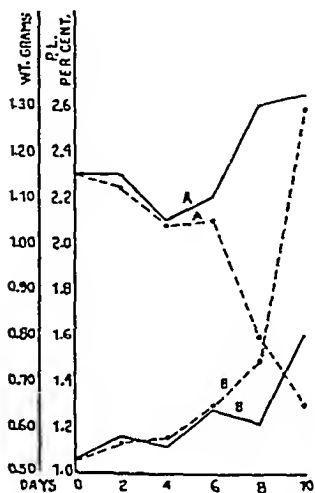


FIG. 1

FIG. 1. Average percentage concentration of phospholipid and average weight of rat kidneys. The solid line indicates the group with the choline supplement; the broken line that without choline. Curves A and B represent phospholipid percentage concentration and kidney weight respectively.

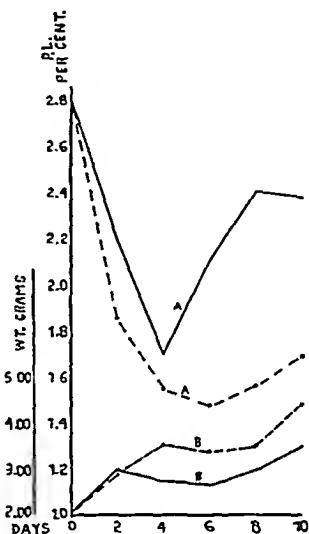


FIG. 2

FIG. 2. Average percentage concentration of phospholipid and average weight of rat liver. The solid line indicates the group with the choline supplement; the broken line that without choline. Curves A and B represent phospholipid percentage concentration and liver weight respectively.

In the case of each series determinations were made of the content of phospholipids in the livers. Fig. 2 shows the results for Series V. It will be noted that, in the group receiving choline, there is an initial, marked decrease in the percentage concentration of liver phospholipids, possibly due to the effect of the transfer of the rats from the stock diet to the experimental, high fat ration. At 6 days the phospholipid concentration had increased. There was, however, a definite difference between the two groups of animals. A similar difference was observed in all series.

DISCUSSION

In confirmation of the work of Welch (6) triethylcholine was found to prevent the production of hemorrhagic kidneys. This substance does not act, according to du Vigneaud, Chandler, Moyer, and Keppel (3), as a donator of methyl groups, but it could be assumed to stimulate the formation of phospholipids, since it has lipotropic activity. More recently, Landau and Welch (9) have shown that arsenocholine will prevent hemorrhagic kidneys and will enter into the synthesis of phospholipids, but will not donate methyl groups to homocystine.

In each of the five series of animals used by us the percentage concentration of phospholipids in hemorrhagic kidneys has been significantly less than in kidneys kept normal by the administration of a lipotropic substance. There was, however, no significant difference between the total amounts of phospholipids in the kidneys. In Series V, on the 8th day, the percentage concentration of phospholipid in the damaged kidneys was 38 per cent less than that in the normal kidneys, while the hemorrhagic kidneys were 23 per cent greater in weight. At this time the total amount of phospholipid in the hemorrhagic kidneys was 24 per cent less. On the 8th day 80 per cent of the rats not receiving choline showed hemorrhage but the marked increase in weight had not yet taken place. It seems significant that the percentage concentration and the total amount of phospholipid had diminished and that hemorrhage had appeared before the kidneys became unusually large.

During the 10 day experimental period the kidneys of the rats given a lipotropic substance show a gradual increase in weight, due apparently to normal growth, and there is an increase in phospholipid percentage concentration. This is in contrast to the changes in the kidneys of the rats not receiving a lipotropic agent. We regard these results as additional evidence in support of the contention of Landau and Welch (9) that renal hemorrhage is not due to the absence of labile methyl groups but is caused by a failure of phospholipid formation. Additional support for their explanation is provided by the data showing phospholipid concentrations in the livers; in animals not receiving choline the phospholipid concentration was definitely less than in the group receiving choline.

SUMMARY

Hemorrhagic kidneys, produced in rats by a deficiency of choline, have a diminished percentage concentration of phospholipids. Renal hemorrhages can be prevented by the administration of triethylcholine as well as by choline. It is suggested, on the basis of the experiments with choline, that the hemorrhages may result from a failure of phospholipid formation in the liver with consequent deficiency in the kidney.

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BIBLIOGRAPHY

1. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
2. Solandt, D. Y., and Best, C. H., *Nature*, **144**, 376 (1939).
3. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
4. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939).
5. Griffith, W. H., and Mulford, D. J., *J. Am. Chem. Soc.*, **63**, 929 (1941).
6. Welch, A. D., *J. Biol. Chem.*, **137**, 173 (1941).
7. Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **56**, 362 (1923).
8. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
9. Landau, R. L., and Welch, A. D., *Federation Proc.*, **1**, pt. 2, 156 (1942).

DISCUSSION

In confirmation of the work of Welch (6) triethylcholine was found to prevent the production of hemorrhagic kidneys. This substance does not act, according to du Vigneaud, Chandler, Moyer, and Keppel (3), as a donator of methyl groups, but it could be assumed to stimulate the formation of phospholipids, since it has lipotropic activity. More recently, Landau and Welch (9) have shown that arsenocholine will prevent hemorrhagic kidneys and will enter into the synthesis of phospholipids, but will not donate methyl groups to homocysteine.

In each of the five series of animals used by us the percentage concentration of phospholipids in hemorrhagic kidneys has been significantly less than in kidneys kept normal by the administration of a lipotropic substance. There was, however, no significant difference between the total amounts of phospholipids in the kidneys. In Series V, on the 8th day, the percentage concentration of phospholipid in the damaged kidneys was 38 per cent less than that in the normal kidneys, while the hemorrhagic kidneys were 23 per cent greater in weight. At this time the total amount of phospholipid in the hemorrhagic kidneys was 24 per cent less. On the 8th day 80 per cent of the rats not receiving choline showed hemorrhage but the marked increase in weight had not yet taken place. It seems significant that the percentage concentration and the total amount of phospholipid had diminished and that hemorrhage had appeared before the kidneys became unusually large.

During the 10 day experimental period the kidneys of the rats given a lipotropic substance show a gradual increase in weight, due apparently to normal growth, and there is an increase in phospholipid percentage concentration. This is in contrast to the changes in the kidneys of the rats not receiving a lipotropic agent. We regard these results as additional evidence in support of the contention of Landau and Welch (9) that renal hemorrhage is not due to the absence of labile methyl groups but is caused by a failure of phospholipid formation. Additional support for their explanation is provided by the data showing phospholipid concentrations in the livers; in animals not receiving choline the phospholipid concentration was definitely less than in the group receiving choline.

SUMMARY

Hemorrhagic kidneys, produced in rats by a deficiency of choline, have a diminished percentage concentration of phospholipids. Renal hemorrhages can be prevented by the administration of triethylcholine as well as by choline. It is suggested, on the basis of the experiments with choline, that the hemorrhages may result from a failure of phospholipid formation in the liver with consequent deficiency in the kidney.

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BIBLIOGRAPHY

1. Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, **35**, 107 (1936).
2. Solandt, D. Y., and Best, C. H., *Nature*, **144**, 376 (1939).
3. du Vigneaud, V., Chandler, J. P., Moyer, A. W., and Keppel, D. M., *J. Biol. Chem.*, **131**, 57 (1939).
4. Griffith, W. H., and Wade, N. J., *J. Biol. Chem.*, **131**, 567 (1939).
5. Griffith, W. H., and Mulford, D. J., *J. Am. Chem. Soc.*, **63**, 929 (1941).
6. Welch, A. D., *J. Biol. Chem.*, **137**, 173 (1941).
7. Steenbock, H., and Nelson, E. M., *J. Biol. Chem.*, **66**, 362 (1923).
8. Bloor, W. R., *J. Biol. Chem.*, **82**, 273 (1929).
9. Landau, R. L., and Welch, A. D., *Federation Proc.*, **1**, pt. 2, 156 (1942).

THE ACTION OF PHENYLTHIOCARBAMIDE ON TYROSINASE

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(Received for publication, July 2, 1942)

Richter and Clisby (1) have investigated the toxic effect of phenylthiocarbamide in rats. The acute toxic effect is characterized by an effusion of fluid into the thoracic cavity, but in chronic poisoning there is a lowered body temperature, which suggests a depression of metabolism, and compensatory changes in the thyroid. It was therefore of interest to test the effect of the drug *in vitro* on isolated enzyme systems.

Further, Richter and Clisby (2) observed graying of the hair in black rats after feeding phenylthiocarbamide to them for 2 or 3 months. This suggested an interference with melanin formation, and for this reason we investigated the effect of the drug on tyrosinase. Phenylthiocarbamide is a very effective inhibitor for tyrosinase, even in concentrations as low as $1 \times 10^{-6} M$, and the characteristics of this inhibition are described. The experiments with animal enzyme preparations have yielded negative results.

EXPERIMENTAL

The tyrosinase of the common mushroom *Psalliota campestris* was used for most of the experiments, but similar results were also obtained with potato tyrosinase. The enzyme was used either as the crude water extract of the mushrooms or partially purified according to the method of Ludwig and Nelson (3). Catechol, *p*-cresol, dihydroxyphenylalanine, tyramine, adrenalin, and tyrosine were used as substrates. The oxidation was measured in the Warburg apparatus at 37°. 0.05 *M* phosphate buffer was used either at pH 6.7 or 7.8. Fig. 1 shows the effect of various concentrations of phenylthiocarbamide on the oxidation of equimolar concentrations of four compounds. In all cases the inhibition is complete at first and then suddenly disappears, and the subsequent rate of oxidation of the substrate is the same as that of the control. The length of the period of complete inhibition varies with the different substrates, and the more rapidly the compound is oxidized the shorter is the period of inhibition by a given concentration of phenylthiocarbamide. Thus catechol which is oxidized very rapidly is inhibited for about 25 minutes by 0.005 mg. of the drug, while tyramine, which under the same conditions is oxidized more slowly, is inhibited for more than 3 hours (see Fig. 1). This type of inhibition could be explained if the phenylthiocarbamide were destroyed by the

enzyme, or if the drug were displaced from the enzyme surface as the oxidation proceeds. However, shaking the phenylthiocarbamide with the purified enzyme for 4 hours before the addition of the substrates does not shorten the inhibition period, as would be expected if the drug were destroyed. To test the validity of the second hypothesis, an experiment was performed with *p*-cresol and is shown in Fig. 2. If the drug is added to the enzyme before the *p*-cresol, the usual inhibition of about 60 minutes is obtained. But if the *p*-cresol is added first and the oxidation is allowed to proceed for 5 minutes before the phenylthiocarbamide is added, very little, if any, inhibition occurs. If the oxidation is allowed to proceed to completion and at that time more *p*-cresol and the drug are added, inhibition

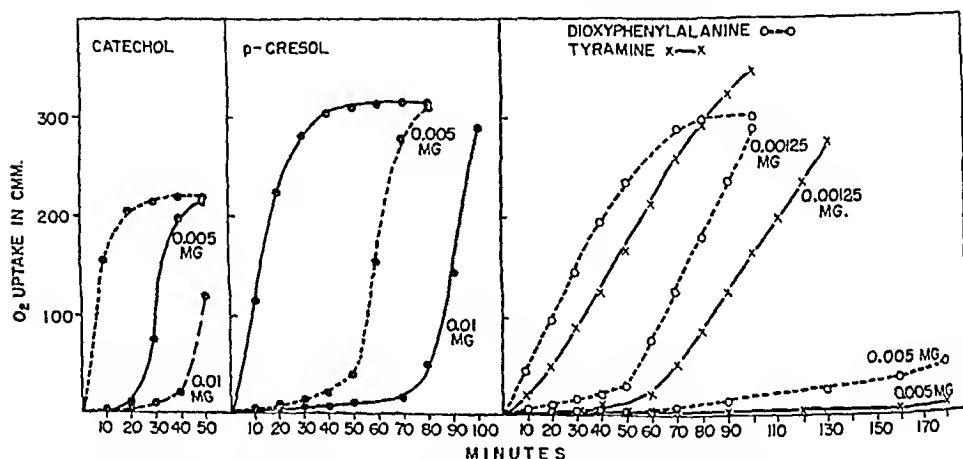


FIG. 1. The effect of various concentrations of phenylthiocarbamide on the oxidation of 1.0 mg. each of the indicated compounds. pH 6.7 and 37°.

again occurs, but for a shorter period. With the dihydroxy compounds, catechol, dihydroxyphenylalanine, and adrenalin, this effect can also be demonstrated. Evidently an intermediate product, and to a lesser extent the end-product, can displace the phenylthiocarbamide from the enzyme surface.

The length of the inhibition period varies with the concentration of the enzyme, the substrate, and the drug. The concentration of the enzyme used was standardized for comparative purposes so that an inhibition of 60 ± 10 minutes was obtained in the presence of 1.0 mg. of *p*-cresol and 3×10^{-5} M phenylthiocarbamide. The inhibition period with a given amount of phenylthiocarbamide is shorter as the concentration of the substrate is increased, indicating that the substrate or its end-product is competing with the drug for the enzyme surface. This is shown in Fig. 3.

The effect of pH on the inhibition period is shown in Fig. 4. Most of the experiments were carried out at pH 6.7 to prevent the autoxidation of the dihydroxyphenylalanine. The inhibition period for *p*-cresol and catechol is, however, longer at pH 7.8 but the rate of oxidation of these compounds is not appreciably affected by this change in hydrogen ion concentration. Phenylthiocarbamide has no effect on the autoxidation of dihydroxyphenylalanine at pH 7.8 nor does it affect the oxidation of cysteine catalyzed by inorganic copper ions.

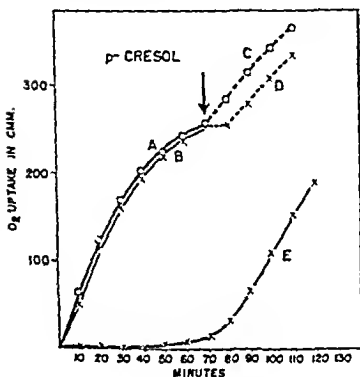


FIG. 2

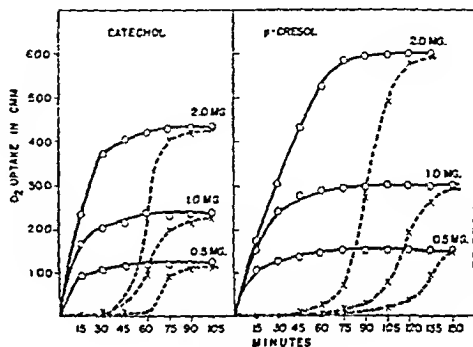


FIG. 3

FIG. 2. Curve A, the oxidation of 1.0 mg. of *p*-cresol. Curve C, at the arrow more *p*-cresol (1.0 mg.) was added to the mixture. Curve B, the oxidation of 1.0 mg. of *p*-cresol; 0.01 mg. of phenylthiocarbamide was added to the mixture 5 minutes after the *p*-cresol. Curve D, at the arrow *p*-cresol (1.0 mg.) with 0.01 mg. of phenylthiocarbamide was added to the mixture. Curve E, 0.01 mg. of phenylthiocarbamide was added to the enzyme before 1.0 mg. of *p*-cresol. pH 6.7 and 37°.

FIG. 3. The effect of 0.01 mg. of phenylthiocarbamide on the oxidation of different concentrations of catechol and *p*-cresol. pH 6.7 and 37°.

Keilin and Mann (4) state that sodium diethyldithiocarbamate inhibits laccase, but they give no details of the inhibition. Fig. 5 shows the effect of this compound on the oxidation of catechol. The type of inhibition is different from that of phenylthiocarbamide in that it increases with time. The lowest concentration of the diethyldithiocarbamate to give a significant inhibition with the standard amount of enzyme is considerably greater than that of phenylthiocarbamide. Moreover, the inhibition period with the latter compound is shorter for catechol than for *p*-cresol, whereas the former is a more effective inhibitor of the oxidation of catechol. The inhibition with thiourea, however, resembles that with phenylthiocarbamide but 10 to 20 times the concentration of thiourea are necessary to

obtain equivalent inhibitions. *p*-Aminobenzoic acid does not protect the enzyme against any of these compounds. Various urethanes even in large concentrations are without effect.

In an attempt to explain the action of phenylthiocarbamide in the rat, we investigated the effect of the drug on certain isolated enzyme systems.

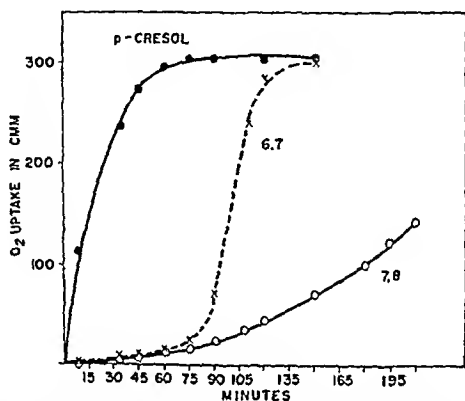


FIG. 4. The effect of the hydrogen ion concentration on the inhibition of the oxidation of 1.0 mg. of *p*-cresol by 0.01 mg. of phenylthiocarbamide. 37°.

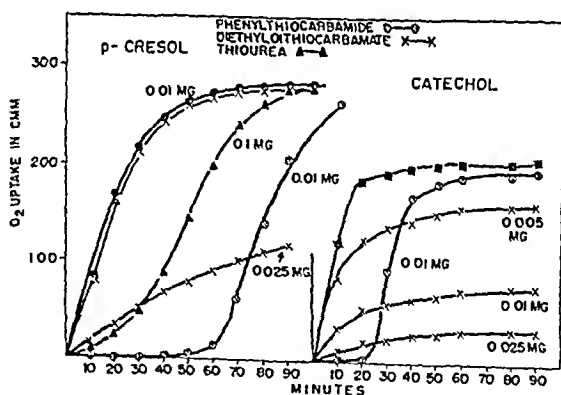


FIG. 5. A comparison of the effects of phenylthiocarbamide, thiourea, and sodium diethyldithiocarbamate on the oxidation of 1.0 mg. each of *p*-cresol and catechol. pH 6.7 and 37°.

Various rat tissues were prepared as slices or broken cell suspensions, but it was found that phenylthiocarbamide even in relatively large concentrations had no immediate effect on the oxygen uptake of such preparations, but caused a small depression after incubation with them for about 3 hours. The tissues used were liver, kidney, brain, and muscle. Investigation of

the effect of the drug on certain specific enzymes also yielded negative results, even after prolonged incubation of the drug with the enzyme. Thus the activity of the cytochrome, succinic acid, choline, *l*-proline, *d*-amino, amine, and sarcosine oxidases of liver and kidney was not affected by a concentration of 50 mg. per cent. The xanthine oxidase of liver was slightly inhibited but the oxidation of glucose, lactic acid, and pyruvic acid by brain was not.

We observed, however, that methemoglobin production, which occurs when broken cell suspensions of liver and kidney are shaken in air (5), is inhibited by phenylthiocarbamide without affecting the oxygen uptake of these tissues. The methemoglobin production has been shown to be caused by H_2O_2 formed when amines, *d*-amino acids, and purines are oxidized (5). The inhibition of the xanthine oxidase may thus partially account for the inhibition of methemoglobin production, but the possibility remains that methemoglobin is formed in part by a copper protein complex which Mann and Keilin (6) have shown to be present in liver and which may be inhibited by the phenylthiocarbamide.

DISCUSSION

Phenylthiocarbamide is a very effective inhibitor of tyrosinase, which is a copper protein enzyme. It has, however, no effect on oxidations catalyzed by inorganic copper. Since, with the possible exception of the dopa oxidase of skin which has not been isolated, copper protein enzymes have not been shown to play any important part in catalyzing oxidations in the mammalian body, the toxic effects of phenylthiocarbamide in the rat are not explained by its action on tyrosinase.

If the dopa oxidase of skin is a copper protein enzyme, it is possible to explain the graying of the hair on the basis of its inhibition by phenylthiocarbamide.

SUMMARY

1. Phenylthiocarbamide is a very effective inhibitor of tyrosinase.
2. The characteristics of the inhibition have been described. It differs from that produced by sodium diethyldithiocarbamate.
3. The significance of this inhibition for the effects produced by phenylthiocarbamide in the animal have been discussed.

BIBLIOGRAPHY

1. Richter, C. P., and Clisby, K. H., *Arch. Path.*, **33**, 46 (1942).
2. Richter, C. P., and Clisby, K. H., *Proc. Soc. Exp. Biol. and Med.*, **48**, 684 (1941).
3. Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2601 (1939).
4. Keilin, D., and Mann, P. J. G., *Nature*, **143**, 23 (1939).
5. Bernheim, F., and Michel, H. O., *J. Biol. Chem.*, **118**, 743 (1937).
6. Mann, P. J. G., and Keilin, D., *Proc. Roy. Soc. London, Series B*, **126**, 303 (1938).

THE SERUM ELECTROLYTES IN THE DOG BEFORE AND DURING ACUTE ALKALOSIS INDUCED BY SODIUM BICARBONATE

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(Received for publication, July 6, 1942)

Although the ingestion of sodium bicarbonate has long been known to produce alkalosis, apparently no detailed study of the electrolyte composition of the blood serum during this disturbance has been recorded in the literature. The present investigation was undertaken to supply this information. The study is of particular interest in demonstrating the unusually severe chemical alterations of the acid-base balance which may be induced in the dog without necessarily resulting in death.

Method of Study

Alkalosis was produced in a 19 kilo dog by intravenous injection of varying quantities of sodium bicarbonate in distilled water at 7 day intervals for a period of 5 weeks. In an additional experiment, a mixture of sodium chloride and sodium bicarbonate was administered to determine the effect of the added chloride on the degree of the alkalosis. Venous blood was withdrawn under oil from the jugular vein before each experiment and at intervals of 5 to 35 minutes after the injection of alkali (see Table I) and utilized for the following determinations: serum carbon dioxide, pH, and chloride (1), calcium (2), phosphorus (3), sodium (4), and total base (5). Measurements were made also of the cell volume and serum water (6). The carbon dioxide tension and the bicarbonate content of the blood were calculated from the CO_2 and pH values by means of the Henderson-Hasselbalch equation.¹ The concentration of electrolytes was calculated also as milliequivalents per kilo of serum water (osmolar concentration), but since no significant additional information was obtained thereby, these data are omitted.

Results

Clinical manifestations during alkalosis consisted of restlessness, retching, and rapid, shallow respirations. In addition, the dog during Experiment V became extremely weak and momentarily exhibited convulsive movements. These symptoms subsided rapidly, however, and the animal

¹ $\text{pH} = \text{pK}' + \log [\text{B} \cdot \text{HCO}_3]/[\text{H} \cdot \text{HCO}_3]$.

TABLE I
Concentration of Electrolytes and Water in Serum of Dog before and during Acute Alkalosis Induced by Sodium Bicarbonate

Experiment No	NaHCO ₃	Distilled water	Injection		pH	pCO ₂	CO ₂	BHCO ₃	Cl	Na	Ca	Total base	P	Serum water		Cell volume		
			Duration	Time of analysis										gm. per l.	gm. per cent			
Control I	27.4	364	60	30	7.42	31.4	20.7	19.76	104.0	148.8	5.55	159.4	1.13	929.9	91.85	49.5		
					7.83	30.5	50.1	49.18	94.4	169.0	4.55	181.0	0.61	935.0	92.11	41.5		
					7.40	33.2	21.3	20.3	104.0	142.7	5.05	154.7	1.20	931.5	91.90	46.2		
Control II	28.1	375	48	35	7.81	30.03	47.2	46.3	97.0	166.4	4.85	178.3	0.70	940.0	92.15	40.0		
					7.39	33.8	21.0	19.98	110.8	144.6	5.45	156.6	1.55	935.8	92.06	47.3		
					7.60	49.5	48.8	47.31	105.0	169.3	5.10	180.2	0.54	932.8	91.58	45.0		
Control III	28.5	285	47	30	7.40	33.5	21.7	20.69	107.2	143.9	5.50	155.0	1.30	936.2	91.55	42.5		
					7.84	36.9	62.0	60.89	101.8	182.5	4.55	192.1	0.45	931.2	91.90	39.5		
					7.41	31.6	22.7	21.75	110.8	143.8	5.40	160.4	0.97	922.4	91.67	43.0		
Control IV	41.3	330	47	20	7.93	37.5	77.8	76.67	100.8	199.0	4.10	209.7	0.76	947.9	93.18	45.0		
					7.42	35.2	23.2	22.24	112.0	147.6	4.88	158.4	0.903	924.2	91.69	46.0		
					7.76	31.0	43.5	42.57	134.8	194.3	4.50	202.3	0.610	943.8	92.28	38.5		
Control V	42.5	340	30	5														
Control VI	23.5*	282	30	12														

* Plus 16.5 gm. of NaCl.

appeared in good condition several hours after the discontinuation of alkali. The onset of clinical improvement coincided usually with the appearance of a marked diuresis. The pH of the urine at this time ranged from 7.6 to 7.95. Tetany was not observed at any time.

The results as shown in Table I indicate the unusual severity of the alkalosis induced in these experiments. The most striking changes in the electrolyte structure consisted of a marked increase in total base, due exclusively to the rise in sodium, and a similar elevation of the bicarbonate content of the blood. There was a slight decrease in the serum chloride and calcium and a more definite lowering of the serum phosphorus. These latter changes are probably attributable to dilution of the blood caused both by the injection of fluid and by the hypertonicity of the sodium bicarbonate solution. The absence of tetany in this dog is probably explained by the fact that the carbon dioxide tension of the blood was not diminished. Shock and Hastings (7) have demonstrated that tetany appears only when the carbon dioxide tension of the blood falls below the critical level of 20 mm. of mercury. The results of Experiment VI clearly show that added sodium chloride did not prevent or diminish the severity of the alkalosis induced by sodium bicarbonate. Indeed, the sodium and total base values were higher than those obtained with comparable amounts of alkali alone; this finding is, of course, to be expected in view of the increased total quantity of sodium ion injected.

SUMMARY

A severe alkalosis can readily be induced in the dog by the intravenous administration of sodium bicarbonate. The acid-base disturbance is characterized by a marked elevation of the sodium and total base and by a similar increase in the bicarbonate content of the blood. The serum chloride, calcium, and phosphorus diminish slightly, probably as a result of the accompanying hydremia.

BIBLIOGRAPHY

1. Peters, J. P., and Van Slyke, D. D., *Quantitative clinical chemistry, Methods*, Baltimore, 283, 796, 835 (1932).
2. Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, **63**, 461 (1925).
3. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
4. Butler, A. M., and Tuthill, E., *J. Biol. Chem.*, **93**, 171 (1931).
5. Stadie, W. C., and Ross, E. C., *J. Biol. Chem.*, **65**, 735 (1925).
6. Hastings, A. B., and Eichelberger, L., *J. Biol. Chem.*, **117**, 73 (1937).
7. Shock, N. W., and Hastings, A. B., *J. Biol. Chem.*, **112**, 239 (1935-36).

ON THE LIBERATION OF FREE AMINO NITROGEN FROM PROTEINS IN THE VAN SLYKE APPARATUS

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This paper reports an investigation of the continued action of nitrous acid on proteins in the Van Slyke apparatus in connection with a simple method for the estimation of lysine.

Kossel (1) and Van Slyke (2) and their coworkers have shown that there is an intimate connection between free amino groups in intact proteins and their lysine content (3). Kossel and Gawrilow (1) as well as Felix (4) have emphasized that one cannot expect a direct proportionality between these two values for reasons which will be discussed below. Block and Bolling (5) suggest that after precipitation of the basic amino acids in a protein hydrolysate with phosphotungstic acid, and decomposition of the phosphotungstates, the free basic amino acids may be treated with NaNO_2 and acetic acid. Whereas the α -amino nitrogen is liberated quantitatively in 5 minutes, the ϵ -amino N of lysine requires 20 minutes. This difference in reaction time permits an estimation of lysine. While it is true that the α -amino N is liberated in such experiments in 5 minutes, it is important to remember that the ϵ -amino N is also liberated to a large extent in the same time and that only a small percentage, changing under different experimental conditions, is developed during the following 15 to 25 minutes. The most important factor here concerned is temperature, as was shown by Sure and Hart (6). We, too, observed that small changes in temperature and other uncontrollable conditions gave rise to considerable variation in the quantity of ϵ -amino N decomposed in the time interval from 0 to 5 and from 5 to 30 minutes, respectively. It was not possible to determine a constant factor correlating the amino N figures found after 5 and after 30 minutes with the lysine content. For these reasons we abandoned the use of hydrolysates and turned to intact proteins. In this connection we came to study the continued action of nitrite and glacial acetic acid on proteins.

EXPERIMENTAL

With an intact protein, only the ϵ -amino group of lysine is thought to react in the Van Slyke apparatus, and the reaction is generally completed in 30 minutes, but there is also a liberation of other amino groups due to the action of the glacial acetic acid on the protein. The amino N decom-

posed in 30 minutes is, therefore, the sum of ϵ -amino N and of an unknown amount of other amino N. In a method designed to determine lysine a correction must be applied for the latter.

In experiments with casein (Hammarsten) extending over several hours with readings every 30 minutes, it was found that the liberation of amino N occurred at a diminishing rate (Table I). This indicates that more amino N is being liberated in the first than in the second half hour period. By plotting the difference in amino N between 30 and 60 minutes (0.24 mg.) and 60 and 90 minutes (0.15) and extrapolating to zero time, one finds a value of 0.33 mg. of N as the correction to be applied to the total amount of N evolved during the first 30 minutes; *i.e.*, $0.89 - 0.33 = 0.56$ mg. of ϵ -amino N. The same result may be arrived at by use of the formula $c - 3(b - a)$, where a , b , and c are, respectively, the amounts of amino N

TABLE I
Rate of Liberation of Nitrogen in Van Slyke Apparatus with 100 Mg. of Casein

Time	Nitrogen liberated	Difference
min.	mg.	mg.
30	0.89	
60	1.13	0.24
90	1.28	0.15
120	1.41	0.13
150	1.52	0.11
180	1.57	0.05
210	1.63	0.06
240	1.69	0.06

found after 30, 60, and 90 minutes. This figure, multiplied by 146/14, gives the amount of lysine in the sample.

We used protein samples of 60 to 80 mg. which were stirred in a small beaker with a few drops of water and then dissolved as quickly as possible in glacial acetic acid. This solvent was preferred to dilute alkali because the latter is slow in dissolving the protein and causes liberation of α -amino groups before the sample can be transferred to the apparatus; moreover, it neutralizes part of the glacial acetic acid, thus slowing down the rate of reaction. The solution is washed at once into the apparatus which already contains glacial acetic acid, NaNO_2 , and caprylic alcohol. Readings are made after 30, 60, and 90 minutes. The obstruction of glass capillaries by protein particles may offer some technical difficulties. In order to be able to use a protein suspension, it is advisable to use an apparatus with wide capillaries.

Table II shows the extent of agreement obtained in independent deter-

minations upon casein. For accurate results it is desirable to take the average of several such determinations. In Table III are shown the results obtained with some typical proteins.

TABLE II
Agreement of Independent Determinations on Casein

Time	Amino nitrogen liberated per 100 mg. casein	Average
<i>min.</i>	<i>mg.</i>	<i>mg.</i>
30 (a)	0.92, 0.91, 0.86, 0.87, 0.94	0.89
60 (b)	1.09, 1.11, 1.16, 1.17	1.13
90 (c)	1.03, 1.41, 1.18, 1.24	1.28

$$c - 3(b - a) = 0.56 \text{ mg. of lysine} = 5.8 \text{ per cent.}$$

TABLE III
Results with Some Proteins

Protein	Average amino nitrogen per 100 mg. protein liberated after			$c - 3(b - a)$	Lysine	Data in literature
	30 min.	60 min.	90 min.			
				<i>mg.</i>	<i>per cent</i>	
Casein (Hammarsten, Merck)	0.89	1.13	1.28	0.56	5.8	6.13 (7) bovine, 5.46 (7) human, 5.9 (5), 7.6 (8), 6.3 (9), 6.25 (10)
Gelatin (Pfanstiehl) .	0.74	0.95	1.09	0.46	4.7	4.6 (5), 5.9 (8,9), 5.3 (11)
Pumpkin seed globulin* .	0.62	0.76	0.86	0.45	4.7	
Gliadin† (Pfanstiehl) . .	0.34	0.52	0.64	0.12	1.25	0.63 (8), 0.92 (9), 0.93 (12)
Zein (Pfanstiehl).....	0.26	0.46	0.59	0	0	

* Prepared by A. White, Yale University.

† Gliadin dissolves in glacial acetic acid with great difficulty; a correction for the quantity not dissolved (about 4 per cent) introduces an uncertainty. Osborne *et al.* (12) assume 1.21 per cent (Van Slyke's N distribution method) to be the maximum and 0.64 per cent (precipitation with picrate) the minimum value; the true value would then be 0.93 ± 0.28 per cent.

When we added lysine to a zein sample, we could not recover more than about 90 per cent of the amino acid; the loss of about 10 per cent is connected with the slower reaction of lysine in the protein medium. Whereas lysine alone gives 100 per cent of its N in 30 minutes, addition of soluble starch (101 mg.) to lysine hydrochloride (10.0 mg.) reduces the yield to 96 per cent.

The figures in Table I for 100 mg. of casein indicate that after 30 minutes (the time for complete evolution of the ϵ -amino N of lysine) further nitrogen is continuously liberated, though at a diminishing rate. If we assume, by extrapolation, the total N evolution for 100 mg. of casein to be 1.75 mg. and subtract from this the figures in Table I (0.89, 1.13 mg., etc.) and finally plot the differences on semilog paper, a straight line is obtained for the "extra amino N" evolved. By extrapolating to zero time we obtain for this extra N an amount of 1.20 mg.; the difference ($1.75 - 1.20 = 0.55$ mg. of N) is the lysine N (see Table II). This finding seems to suggest a mono-

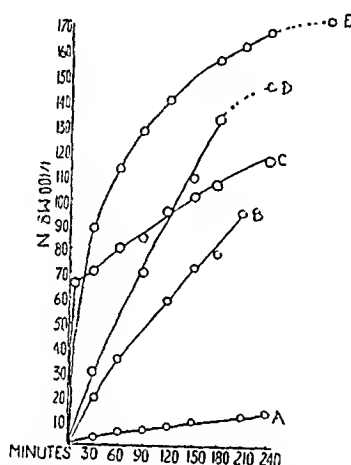


Fig. 1. Liberation of amino nitrogen. Curve A, guanidine nitrate, 5.75 mg., containing 0.66 mg. of amino N in the guanidino group; Curve B, salmine, containing 8.18 mg. of arginine; Curve C, arginine, 8.18 mg., containing 0.66 mg. of amino in the guanidino group; Curve D, zein, 100 mg.; Curve E, casein (Hammarsten), 0 mg.

olecular reaction which occurs in addition to the reaction with lysine during the treatment of the casein with nitrous acid.

As the possible substrate of such a reaction, we examined the reaction of arginine in regard to its guanidino group. Comparing the curve for *D*-arginine (free base, Pfanstiehl; Curve C, Fig. 1) with the curve for an equivalent quantity of guanidine (guanidine nitrate, Pfanstiehl, Curve A), we see that Curve C shows, apart from the liberation of the α -amino N during the first 6 minutes, a much steeper rise than Curve A. With arginine, 77 per cent of the free guanidino amino N is evolved during 4 hours, while in the same time guanidine gives only 20 per cent. This suggests that the side chain in arginine has an effect on the rate of reaction of nitrous acid with the guanidino group. Very similar, but showing a still

steeper rise, is the curve for salmine (salmine sulfate, Squibb; Curve B), a protamine which contains 87.4 per cent arginine but no lysine (13).

The curve for zein (Curve D), which is free from lysine and contains only 1.5 to 1.8 per cent arginine (8), is a fairly straight line up to 3 hours, with slight deviations at 30 and 60 minutes (influence of arginine?). It is safe to assume that here the α -amino N formed by the slow action of the acid plays the dominant rôle.

The casein curve (Curve E), on the other hand, shows a very definite convexity toward the ordinate (after the lysine ϵ -amino N has been liberated). The arginine content, according to Vickery and White (10), is 3.85 per cent. It is evident that the curve for free arginine (Curve C) does not give any clue to the peculiar shape of the casein curve, particularly as it is highly probable that the liberated α -amino groups play their part too. But the evident influence of the side chain in arginine on the rate of reaction of the guanidino amino N, in contrast to the reaction of guanidine itself, suggests that the group to which the guanidino radical is attached would play a still greater part in the case of casein. Thus, the nitrogen, evolved after the complete liberation of the ϵ -amino N of the lysine, may be suspected to arise in part from the arginine of the protein, which thus confers upon the portion of the curve representing the "extra amino N" the characteristics of a monomolecular reaction.

SUMMARY

1. The continued action of nitrous acid on intact proteins in the volumetric Van Slyke apparatus has been studied and it is shown that, by calculation from the quantities of nitrogen liberated after 30, 60, and 90 minutes respectively, an estimate of the quantity of lysine combined in the protein can be obtained.

2. Comparison of the curve for the rate of liberation of nitrogen from casein with those obtained for zein, salmine, arginine, and guanidine suggests that the arginine present in the protein may, by means of partial decomposition of its guanidino group, make the reaction by which extra amino nitrogen is evolved, after liberation of the ϵ -amino nitrogen of lysine, appear to be monomolecular.

BIBLIOGRAPHY

1. Kossel, A., and Gawrilow, N., *Z. physiol. Chem.*, **81**, 274 (1912).
2. Van Slyke, D. D., *J. Biol. Chem.*, **9**, 185 (1911). Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, **16**, 539 (1913-14).
3. Simms, H. S., *J. Gen. Physiol.*, **11**, 629 (1928). Sandstrom, W. M., *J. Physic. Chem.*, **34**, 1071 (1930).
4. Felix, K., *Z. physiol. Chem.*, **110**, 217 (1920).
5. Block, R. J., and Bolling, D., *The determination of the amino acids*, Minneapolis, revised edition (1940).

6. Sure, B., and Hart, E. B., *J. Biol. Chem.*, **31**, 527 (1917).
7. Plimmer, R. H. A., and Lowndes, J., *Biochem. J.*, **31**, 1751 (1937). Cf. also Plimmer, R. H. A., and Rosedale, J. L., *Biochem. J.*, **19**, 1004 (1925).
8. Bodansky, M., *Introduction to physiological chemistry*, New York, 4th edition, 118 (1938).
9. Sherman, H. C., *Chemistry of food and nutrition*, New York, 5th edition, 66 (1937).
10. Vickery, H. B., and White, A., *J. Biol. Chem.*, **103**, 413 (1933). Cf. also Vickery, H. B., and Leavenworth, C. S., *J. Biol. Chem.*, **76**, 707 (1928).
11. Schneider, F., *Collegium* (1940), cf. *Chem. Abstr.*, **34**, 7649 (1940). Cf. also Schryver, S. B., and Buston, H. W., *Proc. Roy. Soc. London, Series B*, **101**, 519 (1927).
12. Osborne, T. B., Van Slyke, D. D., Leavenworth, C. S., and Vinograd, M., *J. Biol. Chem.*, **22**, 259 (1915).
13. Kossel, A., and Dakin, H. D., *Z. physiol. Chem.*, **40**, 311, 565 (1903); **41**, 407 (1904). Cf. also (8).

STUDIES ON BILE ACID METABOLISM

II. THE ACTION OF *ALCALIGENES FAECALIS* ON CHOLIC ACID

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A previous study of the fate of the bile acids in the animal body (1) showed that in the guinea pig cholic acid was converted rapidly to a derivative or derivatives which gave a negative Gregory-Pascoe reaction. This conversion took place principally in the cecum and was brought about by a microorganism identified as *Alcaligenes faecalis*.

The present study was concerned first with some of the factors which regulate the action of *Alcaligenes* on cholic acid. This investigation has shown that the air or oxygen supply of the culture medium, the concentration of cholic acid in the medium, and the number of organisms in the inoculum are important factors in determining the rate of conversion of cholic acid.

This study has also dealt with the reaction products formed by the action of *Alcaligenes* on cholic acid. This investigation has indicated that *Alcaligenes* converts this acid to monoketo-, diketo-, and triketocholanic acids. The end-product of this reaction, 3,7,12-triketocholanic acid, has been isolated from digests in pure form and high yield.

Factors Influencing Conversion of Cholic Acid by Alcaligenes faecalis

Air or Oxygen Supply of Culture Medium—That the conversion of cholic acid by *Alcaligenes* depends upon aeration of the medium is shown by the following experiment. 50 cc. quantities of synthetic medium (2) containing approximately 200 mg. per cent of sodium cholate¹ were placed in each of two 1 liter Erlenmeyer flasks and two 25 × 200 mm. test-tubes. One flask and one tube were inoculated with 1 cc. of an 18 hour infusion broth culture of *Alcaligenes*.² The remaining flask and tube served as controls and were not inoculated. The four vessels were incubated at 37.5° for 96 hours, the flasks being shaken vigorously at 8 hour intervals, whereas the

¹ Sodium cholate used in this and subsequent experiments was prepared from cholic acid (m.p. 197°) supplied for this work through the courtesy of George A. Breon and Company, Kansas City, Missouri.

² The strain of *Alcaligenes faecalis* used throughout this study was isolated from the cecum of a normal guinea pig in November, 1940. Since that time the organism has been transferred daily in beef infusion broth.

tubes were not disturbed. Samples of each reaction mixture obtained prior to incubation and after 24, 48, 72, and 96 hours incubation were analyzed for cholic acid according to the method described previously (1).

The results of this experiment (Table I) show that conversion of cholic acid was considerably more rapid in the well aerated medium in the flask than in the poorly aerated medium in the test-tube. Thus after 48 hours incubation, the medium in the flask contained only 24 mg. per cent of cholic acid, whereas that in the tube contained 145 mg. per cent. It should be mentioned that, during the first 12 hours incubation, the organisms in the test-tube grew less rapidly than those in the flask (the growth was measured turbidimetrically). This, however, did not explain the difference in rate of conversion noted above, for at 24 hours and later incubation periods, the numbers of *Alcaligenes* in the flask and tube were essentially identical.

Additional experiments have shown that oxygen is absolutely essential for the action of *Alcaligenes* on cholic acid. When flasks of medium identi-

TABLE I

Influence of Air Supply on Conversion of Cholic Acid by Alcaligenes

The cholic acid values are given in mg. per cent.

	Cholic acid in medium				
	Prior to incubation	Incubation			
		24 hrs.	48 hrs.	72 hrs.	96 hrs.
1 liter flask, inoculated	169	83	24	19	15
1 " " control	167	169	170	171	170
25 × 200 mm. test-tube, inoculated	169	160	145	132	110
25 × 200 " " control	171	171	170	170	170

cal with those used in the previous experiment were inoculated with *Alcaligenes* and incubated under anaerobic conditions for as long as 14 days, no conversion of cholic acid occurred.

Concentration of Cholic Acid in Medium—That the concentration of cholic acid in the medium is a limiting factor in the rate of conversion of this acid is shown in the following experiment. 50 cc. quantities of medium containing approximately 50, 100, 200, 400, 800, 1200, 2000, and 4000 mg. per cent of sodium cholate were placed in 500 cc. Erlenmeyer flasks and inoculated with 2 cc. of an 18 hour broth culture of *Alcaligenes*. Another 50 cc. portion of medium containing 200 mg. per cent of sodium cholate was not inoculated and served as a control. All nine flasks were incubated at 37.5° for 168 hours, and were shaken at 8 hour intervals. Samples obtained from each flask prior to incubation and after 24, 48, 96, and 168 hours incubation were analyzed for cholic acid.

The results of this experiment (Table II) show that there was prompt and rapid conversion when the concentration of cholic acid in the medium was

367 mg. per cent or less, and no conversion at all when the concentration was 1865 mg. per cent or more. When the medium contained 714 mg. per cent, the reaction lagged during the first 24 hours incubation, but after that proceeded at least as rapidly as in the lower concentrations. A similar lag was noted when the medium contained 1136 mg. per cent of cholic acid, but in this case the reaction was somewhat slower throughout the entire incubation period than that in lower concentrations.

It is noteworthy that these rates of conversion roughly paralleled the growth of *Alcaligenes*. Thus, in media containing 714 and 1136 mg. per cent of cholic acid, growth was less rapid during the first 24 hours than in media containing lower concentrations. When the medium contained 1865 mg. per cent of cholic acid or more, *Alcaligenes* failed to grow.

TABLE II

Action of Alcaligenes on Various Concentrations of Cholic Acid

The cholic acid values are given in mg. per cent.

Flask No.	Cholic acid in medium				
	Prior to incubation	Incubation			
		24 hrs.	48 hrs.	96 hrs.	168 hrs.
1	44	0	0	0	0
2	89	0	0	0	0
3	178	0	0	0	0
4	367	120	0	0	0
5	714	651	328	8	0
6	1136	1066	809	400	60
7	1865	1857	1865	1857	1872
8	3705	3715	3710	3690	3730
Control (not inoculated)	179	179	179	179	179

Number of Organisms in Inoculum—The results of the preceding experiment suggested that the number of organisms present in the digest had a marked effect on the rate of cholic acid conversion. This relation was shown more clearly in the following experiment. 100 cc. quantities of synthetic medium containing 300 mg. per cent of sodium cholate were dispensed into each of four 1 liter Erlenmeyer flasks. Flask 1 was inoculated with 20 cc. of a heavy suspension³ of *Alcaligenes*, Flask 2 with 2 cc., and Flask 3 with 0.2 cc. Flask 4 served as a control. The cholic acid con-

³ The organism suspension was obtained as follows: Infusion agar slants were inoculated with *Alcaligenes* and incubated for 18 hours. The resulting growth was washed from each slant with 2 cc. of synthetic medium, and washings from many slants were pooled. The numbers of organisms in the various inocula were determined by pour plates of suitable dilutions of the organism suspension.

centrations in the various flasks were kept constant by addition of 18 cc. of medium to Flask 2, 19.8 cc. to Flask 3, and 20 cc. to Flask 4. The flasks were incubated at 37.5° for 48 hours and were shaken at 8 hour intervals. Samples obtained from each flask prior to incubation and after 12, 18, 24, and 48 hours incubation were analyzed for cholic acid.

The results obtained after 12 and 18 hours incubation (Table III) show clearly that the larger the inoculum the more rapid the conversion of cholic acid. This relation was particularly evident at 12 hours, when the cholic acid concentrations were 65 mg. per cent in the flask with the largest inoculum, 114 mg. per cent in the flask with the intermediate inoculum, and 164 mg. per cent in the flask with the smallest inoculum.

TABLE III

Influence of Size of Inoculum on Rate of Conversion of Cholic Acid

The cholic acid values are given in mg. per cent.

Flask No.	No. of organisms in inoculum	Cholic acid in medium				
		Prior to incubation	Incubation			
			12 hrs.	18 hrs.	24 hrs.	48 hrs.
1	2×10^{10}	219	65	27	12	0
2	2×10^9	218	114	45	16	0
3	2×10^8	216	164	74	31	0
4	None (control)	216	220	222	224	224

Cholic Acid Derivatives Formed by Action of Alcaligenes

The observation that oxygen was necessary for the action of *Alcaligenes* on cholic acid offered a useful clue to the nature of the reaction. In accord with the theory that cholic acid underwent oxidation, attempts were made to detect oxidation products of this acid in the reaction mixtures. Since mild oxidation of cholic acid by such chemical agents as chromic acid usually results in formation of ketocholic acids, our first experiments were directed toward detection of these keto derivatives in cholic acid digests.

Preliminary Qualitative Experiments—A 500 cc. quantity of synthetic medium containing 300 mg. per cent of sodium cholate was placed in a Fernbach culture flask, inoculated with 25 cc. of a broth culture of *Alcaligenes*, and incubated at 37.5°. Prior to incubation and at 24 hour intervals thereafter, 50 cc. samples of digest were removed from the flask and freed of bacteria by filtration. The filtrates were acidified to Congo red with dilute hydrochloric acid, and the resulting precipitates were filtered off, washed free of mineral acid, and dried at 110°. The cholic acid contents of the dried materials were determined, and in addition tests for keto derivatives were carried out, with the Girard (3) and Zimmermann (4) qualitative reactions.

The precipitate obtained from the digest prior to incubation gave negative Girard and Zimmermann reactions. Precipitates obtained after 24 and 48 hours incubation still contained detectable amounts of cholic acid. These precipitates gave a positive Girard and a negative Zimmermann reaction, indicating that a keto derivative, or derivatives, had been formed and showing that the oxidation had not involved the hydroxyl group at C₃. Precipitates obtained from digests incubated 72, 96, and 144 hours contained no cholic acid, gave positive Zimmermann reactions (increasing in intensity with the time of incubation), and gave stronger Girard reactions than were obtained from 24 and 48 hour digests. These findings showed that the hydroxyl group at C₃ had undergone oxidation and suggested that multiple carbonyl groups had been formed.

Quantitative Study of Formation of Keto Derivatives—Seven 100 cc. quantities of synthetic medium containing 200 mg. per cent of sodium cholate were placed in 1 liter Erlenmeyer flasks, inoculated with 5 cc. of an 18 hour infusion broth culture of *Alcaligenes*, and incubated at 37.5° for various periods up to 15 days. Two control flasks were also incubated for 15 days; one of these contained 100 cc. of the sodium cholate medium, but no organisms; the second contained 100 cc. of plain medium (no sodium cholate) inoculated with *Alcaligenes* as described above.

At the end of the desired period of incubation, 50 cc. of 95 per cent alcohol were added to the contents of a flask; the mixture was heated to boiling, cooled, diluted to 200 cc. with alcohol, and filtered. One aliquot of the filtrate was analyzed for cholic acid. A second aliquot was analyzed for ketocholanic acids according to the Hughes procedure (5). By this method ketosteroids are determined gravimetrically as mercuric iodide hydrazones. The weights of the hydrazones obtained from monoketo-, diketo-, and triketocholanic acids are theoretically 2.486, 3.987, and 5.503 times the weight of the respective keto acids. The equation from which these factors were calculated has been described elsewhere (5). The calculated factors for diketo- and triketocholanic acids have been verified by actual determinations (6).

The results of this experiment have been summarized in Table IV. As shown in Column 6 of Table IV, the weights of mercuric iodide hydrazones obtained from the digests increased with the time of incubation. Thus only 33.3 mg. of hydrazone were obtained from a 24 hour digest, whereas 575 and 910 mg. were obtained from 72 hour and 15 day digests, respectively. This finding is additional proof of the progressive production of carbonyl groups during incubation.

Comparison of the weights of mercuric iodide hydrazones isolated (Column 6) with those that should have been obtained if the cholic acid that disappeared had been converted to monoketo-, diketo-, or triketocholanic acids (Columns 7 to 9) indicated the stepwise conversion of cholic acid to

these keto acids. Thus the amount of hydrazone isolated from the 48 hour digest compared favorably with that required theoretically for conversion of the cholic acid to a monoketocholanic acid, whereas the weight of hydrazone obtained from the 96 hour digest was similar to that required for conversion to a diketo acid. It is especially noteworthy that *the weight of hydrazone obtained from the 15 day digest was almost identical with that required for conversion of all the cholic acid in the medium to triketocholanic acid.*

Attention should be called to another interesting point in Table IV. The mercuric iodide hydrazone obtained from the 24 hour digest was only a fraction of that which should have been found if the cholic acid had been

TABLE IV
Conversion of Cholic Acid to Keto Derivatives by Alcaligenes

Flask No.	Time of incubation	Cholic acid content of medium		Cholic acid converted	Mercuric iodide hydrazone formed	Theoretical amount of mercuric iodide hydrazone formed if cholic acid had been converted to		
		Prior to incubation	At end of incubation			Monoketocholanic acid	Diketocholanic acid	Triketocholanic acid
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	days	mg.	mg.	mg.	mg.	mg.	mg.	mg.
1	1	164	125	39	33.3	97	155	215
2	2	164	9	155	338	385	618	853
3	3	164	0	164	575	408	674	902
4	4	164	0	164	642	408	674	902
5	5	164	0	164	768	408	674	902
6	7	164	0	164	823	408	674	902
7	15	164	0	164	910	408	674	902
Control A*	15	164	164	0	0			
" B†	15	0	0	0	0			

* Containing medium with sodium cholate but not inoculated.

† Containing plain medium inoculated with *Alcaligenes*.

converted to a monoketocholanic acid, 33.3 mg. as compared with 97 mg. An explanation for this finding is not yet available, but it may be either that the monoketocholanic acid does not react quantitatively according to the Hughes-Girard procedure, or that cholic acid is converted to an intermediary substance before being converted to the monoketo acid.

Isolation and Identification of Triketocholanic Acid—Twelve 500 cc. quantities of synthetic medium containing 200 mg. per cent of cholic acid were placed in Fernbach culture flasks, inoculated with *Alcaligenes*, and incubated at 37.5° for 15 days, the flasks being shaken vigorously every 8 hours. At the end of incubation, the digests were pooled and filtered through a Berkefeld candle, yielding 5900 cc. of filtrate. This was con-

centrated under reduced pressure to 1 liter volume, made alkaline to phenolphthalein with dilute ammonium hydroxide, poured on ice, and acidified with dilute hydrochloric acid. The resulting acid precipitate was redissolved in dilute ammonium hydroxide, diluted to 1 liter with iced water, and reprecipitated with dilute hydrochloric acid. Upon standing, this precipitate became crystalline; the crystals were filtered off, washed free of mineral acid, and dried at 110°; yield 10.0 gm. Concentration of the mother liquor yielded an additional 1.2 gm. of amorphous acid, which was not purified further.

The crystalline acid melted at 210–218°, and gave a strongly positive Zimmermann reaction and negative Gregory-Pascoe and Pettenkofer reactions. According to the Hughes procedure, three carbonyl groups were present. The neutralization equivalent showed that the acid had one carboxyl group. Two recrystallizations of the acid from absolute ethyl alcohol gave 7.8 gm. of crystalline acid (m.p. 236–237°); this melting point was not changed by two additional recrystallizations. Comparison of this recrystallized acid with a highly purified sample of 3,7,12-triketocholanic acid indicated that the two compounds were identical. The melting points were the same (236–237°) and there was no depression of the melting point when these acids were mixed. Both compounds formed methyl esters melting at 239–241°; the mixed melting points of these esters showed no depression.

Comment

The data above have shown that cholic acid is oxidized by *Alcaligenes faecalis* to 3,7,12-triketocholanic acid. When the factors that limit the activity of the organism are adequately controlled, this reaction is highly efficient, giving at least an 83 per cent yield of relatively pure triketocholanic acid. The purity of this product and the ease with which it can be obtained may make oxidation of cholic acid by *Alcaligenes* preferable to oxidation with chromic acid.

Although at present only 3,7,12-triketocholanic acid, the end-product of the reaction, has been isolated from digests of cholic acid, quantitative studies have indicated that at least two other oxidation products are formed as intermediaries; these are probably monoketo- and diketocholanic acids. Theoretically one could obtain three monoketo- and three diketocholanic acids; namely, 3-keto-7,12-dihydroxychoholic, 7-keto-3,12-dihydroxychoholic, 12-keto-3,7-dihydroxychoholic, 3,7-diketo-12-hydroxychoholic, 3,12-diketo-7-hydroxychoholic, and 7,12-diketo-3-hydroxychoholic acids. The compounds having a carbonyl group at C₃ may be tentatively eliminated from consideration, since crude acids obtained from

digests incubated for short periods gave negative Zimmermann reactions. It seems likely therefore that the monoketo acid formed in the oxidation by *Alcaligenes* is either 7-keto-3,12-dihydroxycholanic or 12-keto-3,7-dihydroxycholanic acid, whereas the diketo acid is probably 7,12-diketo-3-hydroxycholanic acid. Experiments on the isolation and identification of these compounds are now in progress.

SUMMARY

Study of the action of *Alcaligenes faecalis* on cholic acid has shown the following.

1. The action of *Alcaligenes* on cholic acid is considerably more rapid in a well aerated medium than in a poorly aerated medium. Oxygen is essential for the reaction.

2. High concentrations of cholic acid in the culture medium inhibit the growth of *Alcaligenes*. Consequently, the rate at which this organism attacks cholic acid varies inversely with the concentration of the acid in the medium; the reaction is relatively rapid when the cholic acid concentration is 1136 mg. per cent or less, and is blocked completely when the concentration is 1865 mg. per cent or more.

3. The larger the number of *Alcaligenes* in the inoculum, the more rapid the disappearance of cholic acid from the medium.

Study of the nature of the reaction has shown that *Alcaligenes* oxidizes the hydroxyl groups of cholic acid to carbonyl groups. Both qualitative and quantitative experiments have indicated that the three hydroxyls of cholic acid are attacked in a stepwise manner; qualitative experiments have shown that the hydroxyl located at C₃ is the last to undergo oxidation.

The end-product of the action of *Alcaligenes* on cholic acid has been identified as 3,7,12-triketocholanic acid. This compound has been isolated from suitable digests in pure form and in 83 per cent of the theoretical yield.

BIBLIOGRAPHY

1. Schmidt, L. H., and Hughes, H. B., *J. Biol. Chem.*, **143**, 771 (1942).
2. Sahyun, M., Beard, P., Schultz, E. W., Snow, J., and Cross, E., *J. Infect. Dis.*, **58**, 28 (1936).
3. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).
4. Zimmermann, W., *Z. physiol. Chem.*, **233**, 257 (1935).
5. Hughes, H. B., *J. Biol. Chem.*, **140**, 21 (1941).
6. Hughes, H. B., *J. Biol. Chem.*, **143**, 11 (1942).

THE METABOLISM OF PYRUVATE BY LIVER FROM PANTOTHENIC ACID- AND BIOTIN-DEFICIENT RATS*

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The close relationship of several vitamins of the B complex to respiratory enzymes (1) suggests the possibility that other vitamins of this group may function in a similar manner. There is evidence that pantothenic acid and biotin affect the respiration of unicellular organisms and plant tissue. Pratt and Williams (2) have reported that pantothenic acid stimulated the respiration of yeast and of apple and potato tissue as well as the fermentation of yeast maceration juice. Dorfman *et al.* (3) found that pantothenic acid increased the oxidation of pyruvate by the organism *Proteus morganii* grown on a pantothenic acid-deficient medium. Similarly, Burk *et al.* (4) observed that biotin stimulated the fermentation and respiration of yeast grown on a biotin-low medium.

This report is concerned with similar studies on animal tissues; namely, the ability of liver tissue from pantothenic acid- and biotin-deficient rats to oxidize various substrates, particularly pyruvate.

EXPERIMENTAL

Care of Animals—21 day-old, male, albino rats were given purified rations designed to produce pantothenic acid and biotin deficiencies, respectively. The rations and the vitamin supplements used are given in Table I. The deficient groups were fed *ad libitum*. The food consumptions for the two control groups were restricted to amounts which limited their growth rates to those of the respective deficient groups. The minimum was 3 gm. of ration per day. All rats were maintained on these rations for 8 to 12 weeks. At this time the deficient animals exhibited the typical pantothenic acid and the biotin deficiency syndromes respectively. No pathological manifestations were observed in the control rats.

Respiration Studies—The animals were fasted for 24 hours, decapitated, and the livers removed and blotted between moistened filter paper. A 10 per cent liver homogenate (6) was prepared in the ice-cold buffer solution. 1 ml. of this suspension, containing 100 mg. of wet weight of tissue, was placed in each flask.

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The Ringer-phosphate buffer solution, pH 7.4, (no calcium), described by Feinstein and Stare as *Medium 2* (7) was used in a final phosphate concentration of M/60. The substrates were the sodium salts of pyruvic (Eastman), fumaric (Kahlbaum), and *l*(+)-lactic¹ acids. Aqueous solutions of the fumaric and lactic acids were neutralized to pH 7.2 with dilute sodium hydroxide solution. The pyruvic acid was distilled at 2 to 4 mm. of Hg, dissolved in water, and carefully neutralized to pH 6.8. These substrates were added to the flasks to give a final concentration of 0.03 M.

TABLE I
Purified Rations Employed

	Pantothenic acid-deficient	Biotin-deficient	Controls
Constituents			
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Sucrose .	73	73	73
Casein (Labco).	18	8	18
Dried egg albumin		10	
Salts 4 (5)	4	4	4
Corn oil .	5	5	5
Daily supplements*			
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
Thiamine hydrochloride	0.020	0.020	0.020
Pyridoxine hydrochloride	0.025	0.025	0.025
Nicotinic acid	0.025	0.025	0.025
Riboflavin	0.040	0.040	0.040
Calcium pantothenate		0.100	0.100
Choline hydrochloride	10.0	10.0	10.0

We are indebted to Merek and Company, Inc., Rahway, New Jersey, for generous supplies of thiamine, pyridoxine, nicotinic acid, riboflavin, pantothenic acid, and choline, and to the Abbott Laboratories, North Chicago, Illinois, for the haliver oil used in this study.

* Fed in a supplement dish in 1 ml. of 10 per cent alcohol solution. Each rat was also given 2 drops of haliver oil per week.

All experiments were conducted in the Barcroft differential respirometer at 37° in a total volume of 3 ml. The center well contained 0.3 ml. of a 10 per cent potassium hydroxide solution and a strip of filter paper. The gaseous phase was air. After a 10 minute equilibration period, readings were taken every 10 minutes for 1 hour.

The effects of various possible intermediary catalysts and of several

¹ We wish to express our thanks to Mr. H. J. Koepsell for supplies of pure *l*(+)-lactic acid, prepared by fermenting glucose with *Lactobacillus casei*.

levels of tissue were tested in an effort to increase the respiration due to the individual substrates. Coenzyme I, cocarboxylase, and muscle adenylic acid, each at a level of 20 γ per flask, and cytochrome *c* at 2×10^{-8} mole per flask were tried. None of these had any appreciable effect on the respiration in the presence of pyruvate. Coenzyme I raised the Q_{O_2} due to lactate and fumarate and was, therefore, always used with these two substrates and their blanks.

Results

The results of the respiration in the presence of pyruvate are summarized in Table II. Because the values for each series of controls fell within the same range, they are included under one heading in Table II. The Q_{O_2} (pyruvate) is considerably lower in each series of deficient rats than in the controls during the first 20 minutes. However, because the endogenous uptake was often high during this interval, it is felt that the

TABLE II
Pyruvate Oxidation by Liver Homogenates from Pantothenic Acid- and Biotin-Deficient Rats

Group (12 rats in each)	Q_{O_2} (pyruvate),* 0-20 min.	Q_{O_2} (pyruvate), 20-40 min.
Pantothenic acid-deficient	2.20 (-0.2- 4.6)	0.98 (-0.3- 1.6)
Biotin-deficient.	2.23 (0.4- 3.9)	1.40 (0.3- 3.1)
Controls.	6.48 (2.8-10.4)	8.50 (4.9-13.1)

* Q_{O_2} (pyruvate) is the c.mm. of O_2 consumed per mg. of dry weight of tissue per hour in the presence of pyruvate minus the endogenous Q_{O_2} . Average Q_{O_2} values are given, with the range of values in parentheses.

values during the second 20 minute period more nearly give an indication of the true enzymatic activity under the conditions of the experiment. Results based on these values make it even more evident that tissues from both deficient groups are low in some components of the enzymatic systems concerned in the utilization of pyruvate. Preliminary evidence indicates that pyruvate removal by liver tissue is also diminished in these deficiencies.

The rate of respiration in the presence of fumarate was comparable in the three series during both the first and second 20 minute periods. Oxygen uptake in the presence of lactate for each deficient group fell within the normal range for the first 20 minutes. A lowered Q_{O_2} (lactate) for the second 20 minute interval, noted with some deficient animals, may be attributed to the decreased oxidation of the pyruvate formed.

The presence of certain 4-carbon dicarboxylic acids is known to be necessary for the oxidation of pyruvate by dialyzed brain tissue (8). Under our experimental conditions, the addition of fumaric and malic

acids in a concentration of 0.0003 M did not increase pyruvate oxidation, indicating that, if they are necessary, they are already present in sufficient quantities. 12 γ of sodium pantothenate² added to flasks containing tissue from pantothenic acid-deficient rats did not alter the oxygen uptake in the presence of pyruvate during a period of 1 hour.

DISCUSSION

Many pathways for the removal of pyruvate are known (9). Our results clearly indicate that one or more of these systems involved in the metabolism of pyruvate are affected by both pantothenic acid and biotin deficiencies in rats. These results may be taken as indirect evidence that these two vitamins either are components of these systems or are closely connected with their formation. Work is now in progress to define the specific systems which are deranged in the vitamin deficiencies.

Other investigations have shown³ that the evaluation of enzymatic measurements conducted on liver from animals maintained on a controlled feeding experiment is complicated by the factor of partial starvation. However, the changes in rate of pyruvate oxidation observed in our experiments are entirely too large for their significance to be invalidated by the partial starvation of the controls.

SUMMARY

1. A decreased rate of pyruvate oxidation was observed in liver from pantothenic acid- and biotin-deficient rats.
2. These results indicate, indirectly, that these vitamins may be components of the enzyme systems concerned with the metabolism of pyruvate.

BIBLIOGRAPHY

1. Alexander, J., in A symposium on respiratory enzymes, Madison (1942).
2. Pratt, E. F., and Williams, R. J., *J. Gen. Physiol.*, **22**, 637 (1939).
3. Dorfman, A., Berkman, S., and Koser, S. A., *Federation Proc.*, **1**, pt. 2, 108 (1942).
4. Burk, D., Winzler, R. J., and du Vigneaud, V., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. xxi (1941).
5. Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 459 (1941).
6. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
7. Feinstein, R. N., and Stare, F. J., *J. Biol. Chem.*, **135**, 393 (1940).
8. Banga, I., Ochoa, S., and Peters, R. A., *Biochem. J.*, **33**, 1980 (1939).
9. Stern, K. G., in Luck, J. M., Annual review of biochemistry, Stanford University, **9**, 1 (1940).

² We are grateful to Mr. A. L. Neal who supplied us with the synthetic sodium pantothenate.

³ Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **145**, 297 (1942).

AVAILABILITY OF FORMYLGLYCINE, ACETYLGLYCINE, AND PROPIONYLGLYCINE FOR THE SYNTHESIS OF HIPPURIC ACID BY THE RABBIT*

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Observation of the effect on the rate of excretion of hippuric acid formed when glycine or a glycine derivative is administered simultaneously with benzoic acid to rabbits has been used in the study of possible conversion of certain N-alkylglycine derivatives to glycine in the rabbit (1, 2). The results of similar experiments with certain N-acyl derivatives of glycine are presented here.

Interest in N-acylamino acids has been centered around the acetyl derivative and has been stimulated recently by the work of du Vigneaud and coworkers (3, 4), who a few years ago reestablished on firmer ground Knoop's acetyl theory of amino acid synthesis. According to this hypothesis, the acetylamino acid may be an important intermediate in the *in vivo* reactions of amino acids. Acetylglycine might thus be an intermediate in the *in vivo* synthesis of glycine. Also, in the case of glycine, the formyl and acetyl derivatives are of interest in the light of evidence obtained in several laboratories indicating that, while acetyl and formyl derivatives of certain *l*-amino acids were utilized by an animal, the derivatives of the corresponding *d* isomer were not utilized, even though the *d*-amino acid itself might be converted to the *l* form in the body (5). It is thus of interest to know whether the formyl and acetyl derivatives of glycine, an amino acid which does not exist in stereoisomeric forms, will be utilized by the organism as are the acetyl and formyl derivatives of certain *l*-amino acids, or whether they will be non-utilizable as are corresponding derivatives of *d*-amino acids. In regard to other amino acids, it has been found that the formyl derivatives of *l*-methionine and *l*-cystine and the acetyl derivatives of *l*-homocystine, *dl*-phenylalanine, *l*-tryptophane, and *l*-cystine could replace the corresponding unsubstituted amino acid for purposes of growth in the diet of the young white rat (5). Apparently the only propionyl derivative studied heretofore is propionyltryptophane (6). It likewise could replace the unsubstituted amino acid for rat growth.

EXPERIMENTAL

Formylglycine was prepared from glycine by formylation with the mixed anhydride of formic and acetic acids (7). Acetylglycine was prepared as

* Presented at the Thirty-sixth annual meeting of the American Society of Biological Chemists at Boston, April, 1942 (*Federation Proc.*, 1, pt. 2, 97 (1942)).

described by Herbst and Shemin (8). Purity of these compounds was established by melting point, nitrogen analysis, and neutral equivalent titration. Propionylglycine, which has not been described previously, was prepared in the following manner. To 20 gm. of dry, powdered glycine in a 125 cc., glass-stoppered Erlenmeyer flask 40 cc. of propionic anhydride were added. The mixture was warmed in a water bath at about 95° with occasional shaking for about 5 minutes or until the reaction began. At this point the glycine dissolved and the solution began to turn yellow. The flask was immediately cooled under running water. The resulting orange solution began to crystallize on cooling and was put in the refrigerator overnight. The solid mass obtained was transferred to a Buchner funnel and dried by suction. The yellow crystalline material was washed with ice-cold water and recrystallized from water until colorless and free from the odor of propionic acid. White, odorless crystals melting at 126-127° (corrected) were obtained. Nitrogen found, 10.73, 10.77 per cent; theoretical, 10.68 per cent. Titration with 0.1 N sodium hydroxide with the use of phenolphthalein as an indicator gave neutral equivalents, 129.0, 129.3; theoretical, 131.1.

The experimental procedure and methods of analysis have been described in detail in a previous study (1). Male rabbits weighing from 2 to 3 kilos were used. Benzoic acid was given at a constant level, 0.66 gm. per kilo of body weight, and the glycine or derivatives at a level of 3 molecular equivalents of the amount of benzoate fed. The sodium benzoate, alone, with glycine, or with one of the derivatives under investigation, was given in solution by stomach tube. The N-acylamino acid in solution was made neutral to litmus before addition of sodium benzoate, and both compounds, in solution, were thus administered simultaneously. 6 and 18 hour urine specimens were collected, the bladder being emptied completely by gentle pressure on the abdominal wall.

The *rate of excretion* is shown by the percentage of administered benzoic acid which is excreted in the first 6 hours. Values for the succeeding 18 hour periods indicate only completeness of the recovery of the administered benzoic acid and are not included in Table I, since we are concerned primarily with *rate* of excretion as an index of availability of glycine. Creatinine determinations were made to check completeness of urine collections. These values, as well as those for total nitrogen, are also omitted, as they do not bear directly on this study.

DISCUSSION

In Table I are presented data showing the effect on the rate of excretion of hippuric acid when glycine or one of the N-acylglycine derivatives is administered simultaneously with sodium benzoate. The data are from consecutive experiments at least 5 days apart. Control experiments with

TABLE I

Excretion of Benzoic Acid, Total and As Hippuric Acid, in 6 Hour Period Immediately Following Ingestion of Sodium Benzoate Alone and with Glycine or N-Acylglycine Derivatives

Rabbit No.	Benzoic acid excreted as				Compound fed*	Benzoic acid given
	Hippuric acid		Total			
	gm.	per cent intake	gm.	per cent intake		gm.
6	0.555	33.3	0.819	49.1	None	1.668†
	0.994	59.6	1.249	74.9	Acetyl glycine	1.668
	0.556	31.2	0.941	52.8	None	1.782
	0.647	36.3	1.024	57.4	"	1.782
	1.094	61.3	1.363	76.4	Acetyl glycine	1.782
	0.416	23.3	0.804	45.1	None	1.782
	1.234	69.2	1.455	81.6	Glycine	1.782
	0.359	19.8	0.662	36.4	None	1.818
	0.674	37.1	0.974	53.6	Formyl glycine	1.818
	0.455	25.0	0.751	41.3	None	1.818
	0.787	43.3	1.125	61.9	Formyl glycine	1.818
	0.409	22.5	0.640	35.2	None	1.818
	0.805	44.3	1.197	65.8	Propionyl glycine	1.818
	0.893	49.2	1.219	67.0	"	1.818
	0.930	51.2	1.197	65.8	"	1.818
	0.542	29.8	0.809	44.5	None	1.818
	0.426	23.4	0.700	38.5	Sodium chloride‡	1.818
5	0.820	48.6	1.051	62.3	None	1.689
	0.954	54.7	1.126	64.5	"	1.743
	1.192	68.4	1.490	85.5	Acetyl glycine	1.743
	0.762	42.3	0.982	54.5	None	1.800
	1.264	70.2	1.415	78.6	Acetyl glycine	1.800
	1.405	78.0	1.500	83.3	Glycine	1.800
	0.617	34.0	0.834	45.8	None	1.818
7	1.069	58.8	1.335	73.5	Formyl glycine	1.818
	1.147	70.2	1.227	75.1	Glycine	1.632
	0.604	37.0	0.823	50.3	None	1.632
	0.832	50.9	1.018	62.3	Formyl glycine	1.632

* 3 molecular equivalents of the amount of benzoic acid administered were given. The N-acylamino acid in solution was made neutral to litmus before addition of sodium benzoate.

† The benzoic acid, 0.66 gm. per kilo of body weight, was administered orally as the sodium salt. Since the animal increased in weight, the absolute amount of benzoic acid fed increased as the experiments progressed.

‡ 6 gm. of sodium chloride were given with benzoate in order to produce marked diuresis.

benzoate alone precede or follow experiments with the compounds under study, and comparison of the effects of glycine or glycine derivatives should be made with the nearest control.

From the figures showing the percentage of administered benzoic acid excreted as hippuric acid in the 6 hour period, it can be seen that the simultaneous administration of formylglycine, acetylglycine, or propionylglycine in every case definitely increased the rate of excretion of hippuric acid. The effect of these derivatives, although marked, was not as great as that of glycine itself. The effect of formylglycine on the rate of excretion of hippuric acid after benzoate administration appears to be less than that of acetylglycine. This might be due to slower hydrolysis of formylglycine. That acetylglycine is hydrolyzed more readily than formylglycine by extracts of rabbit liver or kidney is suggested by the experiments of Kimura (9). The present experiments, however, are not adequate to show small differences in the degree of effectiveness among the derivatives used. The increase in rate of excretion of hippuric acid was also reflected in the increased output of total benzoic acid in the 6 hour period. That the increased rate of excretion was not due to diuresis is shown in the experiment in which 6 gm. of sodium chloride were administered with benzoate. A marked increase in urine volume occurred with no increased excretion of hippuric acid or total benzoic acid. Experiments with other compounds which did not increase the rate of excretion of hippuric acid (betaine and N,N-dimethylglycine) also show that diuresis is not the cause of increased excretion of hippuric acid in the 6 hour period (1).

Since the determination of hippuric acid by the Griffith method (10) is not specific, in that it is a determination of nitrogenous compounds in urine extracted by ether from acid solution and not destroyed by sodium hypobromite, it was necessary to be sure that the increases in hippuric acid and total benzoic acid noted in these experiments were not the result of appearance in the urine of substances (either unchanged N-acylglycine derivative or a metabolite) interfering with the analytical procedures. This was of especial importance since *in vitro* experiments showed that high values for hippuric acid were obtained when solutions containing 4 times more N-acylglycine nitrogen than hippuric acid nitrogen were analyzed by the Griffith procedure. This interference increased with increasing molecular weight of the N-acylglycine derivative. When analyzed for total benzoic acid by the method of Kingsbury and Swanson (11), however, those solutions containing formylglycine or acetylglycine gave close to theoretical values for the benzoic acid contained (as hippuric acid), whereas the presence of propionylglycine led to high results. Hence, formylglycine and acetylglycine, although interfering with the hippuric acid determination in pure solutions, did not interfere with the determination of total benzoic acid, whereas propionylglycine interfered with both. Because of these findings control experiments were run in which the same amount of N-acylglycine derivative as was given simultaneously with benzoate to Rabbit 6 (formylglycine 4.6 gm., acetylglycine 5.24 gm., propionylglycine

5.86 gm.) was given alone to the same rabbit. It was found that formylglycine or acetylglycine administered alone did not increase the hippuric acid or total benzoic acid values obtained for the small, constant amount of benzoic acid (about 18 mg.) which is normally eliminated in a 6 hour period by the rabbit. In the animal experiments, therefore, the administration of this amount of formylglycine or acetylglycine did not cause excretion in the urine of substances interfering with the analytical procedures used. The administration of propionylglycine alone, however, did increase the apparent hippuric acid and total benzoic acid excretion in the 6 hour period after administration. The increase over the normal elimination in the 6 hour period caused by this derivative amounted to 94 mg. (calculated as benzoic acid) and affected both hippuric acid and total benzoic acid determinations. For this reason approximate values for benzoic acid excreted as hippuric acid and total benzoic acid in experiments with propionylglycine (Table I) have been obtained by subtracting 100 mg. from the values obtained on urine analysis. The data presented for experiments with propionylglycine are therefore not as accurate as those obtained in the experiments with formylglycine and acetylglycine. They indicate, however, that the amount of benzoic acid excreted in the 6 hour period as hippuric acid and as total benzoic acid in experiments with propionylglycine is still much greater than when benzoate alone was given.

SUMMARY

The preparation of N-propionylglycine is described.

N-Formylglycine, N-acetylglycine, and N-propionylglycine when administered orally with benzoate to rabbits increased the rate of excretion of hippuric acid. The effect of these derivatives, although marked, was not as great as that of glycine.

The availability of these compounds for the synthesis of hippuric acid is interpreted as evidence of their conversion to glycine by the rabbit.

BIBLIOGRAPHY

1. Abbott, L. D., Jr., and Lewis, H. B., *J Biol Chem*, **131**, 479 (1939).
2. Abbott, L. D., Jr., and Lewis, H. B., *J Biol Chem*, **137**, 535 (1941)
3. du Vigneaud, V., and Irish, O. J., *J Biol Chem*, **122**, 349 (1937-38)
4. du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J Biol Chem*, **131**, 273 (1939)
5. Jackson, R. W., and Chandler, J. P., in Luck, J. M., Annual review of biochemistry, Stanford University, **8**, 249 (1939)
6. Berg, C. P., and Hansen, H. E., *Proc Iowa Acad Sc*, **41**, 165 (1934)
7. Bilmann, E., Jensen, K. A., and Jensen, H. B., *Bull Soc chim, Mém*, **1**, 1664 (1934).
8. Herbst, R. M., and Shemin, D., in Johnson, J. R., et al., Organic syntheses, New York, **19**, 4 (1939).
9. Kimura, H., *J. Biochem*, Japan, **10**, 207 (1929)
10. Griffith, W. H., *J Biol Chem*, **69**, 197 (1926)
11. Kingsbury, F. B., and Swanson, W. W., *J Biol Chem*, **48**, 13 (1921)

Abstract

THE INFLUENCE OF SUBSTRATE STRUCTURE ON THE KINETICS OF CARBOXYPEPTIDASE ACTION

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(Received for publication, July 2, 1942)

When the proteolytic enzymes are classified according to their specificity, the group of carboxypeptidases is found to contain several enzymes of similar specificity, such as the carboxypeptidase of beef pancreas or the carboxypeptidase of swine kidney (1). The pancreatic carboxypeptidase acts optimally at about pH 7.7 and is not activated by sulfhydryl compounds, while the kidney enzyme is activated by sulfhydryl compounds and acts optimally near pH 5.4. The similarity in the specificities of these enzymes is evidenced by the fact that both have been found to split the substrate carbobenzoxyglycyl-*l*-phenylalanine about 1.8 times as fast as the substrate carbobenzoxyglycyl-*l*-tyrosine.¹ Such enzymes, belonging to the same specificity group, are termed "homospecific" (2). A group of homospecific trypsinases and a group of homospecific pepsinases have also been described.

It is a general experience that the rate at which a proteolytic enzyme acts upon a substrate of known structure is a function of (a) the nature of the enzyme and (b) the structural details of the substrate. In this communication it is assumed that the rate of the action of a proteolytic enzyme is related to substrate structure by the following equation.

$$K = p_E [E] \cdot a \cdot b \cdot r \quad (1)$$

K is the reaction velocity constant, p_E is a proportionality factor characteristic of the enzyme E , and $[E]$ is the enzyme concentration (in mg. of protein nitrogen per cc. of test solution). a , b , and r characterize numerical factors determined by the structure of groupings A , B , and R in the substrate molecule. Illustrations of these groupings in substrates for trypsinases, pepsinases, and carboxypeptidases are presented in Fig. 1.

Thus R is defined as an amino acid residue bearing the side chain which is essential for enzyme action (Equation 1); A represents the substituent attached to the α -imido group of R ; and B represents the group attached to

¹ Recent experiments (Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., unpublished data) have shown that near pH 5 small differences in pH greatly influence the speed of the action of cysteine swine kidney carboxypeptidase. When the hydrolysis of carbobenzoxyglycylphenylalanine and carbobenzoxyglycyltyrosine by this enzyme is compared at identical pH values, the quotient $CCGlyP/CCGlyT$ is about 1.4.

the α -carbonyl group of R . The arrow denotes the peptide linkage hydrolyzed by the enzyme.

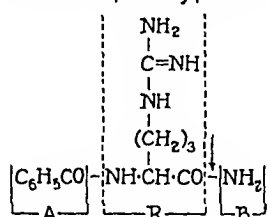
In a previous paper (3) of this series, the proteolytic coefficient C was defined as the rate of the action of a proteolytic enzyme upon its substrate when the enzyme concentration is 1 mg. of protein nitrogen per cc. of test solution, as expressed in Equation 2.

$$C = K/[E] \quad (2)$$

Consequently, Equation 1 may be transformed into Equation 3.

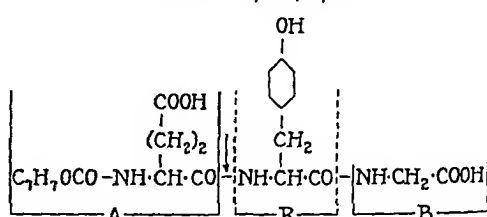
$$C = p_E \cdot a \cdot b \cdot r \quad (3)$$

Substrate for trypsinases



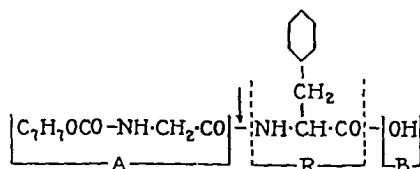
Benzoyl-L-arginine amide

Substrate for pepsinases



Carbobenzoxyl-L-glutamyl-L-tyrosyl glycine

Substrate for carboxypeptidases



Carbobenzoxylglycyl-L-phenylalanine

FIG. 1

The absolute values of the terms p_E , a , b , and r cannot be determined and the validity of Equation 3 cannot therefore be tested directly. It is possible, however, to test several consequences of Equation 3 by one of the following four procedures: (a) by comparing the action of an enzyme upon two substrates which differ only with respect to their R groups, (b) by comparing the action of an enzyme upon two substrates which differ only with respect to their A groups, (c) by comparing the action of an enzyme upon two substrates which differ only with respect to their B groups, (d) by comparing the action of two homospecific enzymes upon the same substrate.

Procedure (a) was applied to the hydrolysis by pancreatic carboxypeptidase of the two substrates, carbobenzoxylglycyl-L-phenylalanine

(CGlyP) and carbobenzoxyglycyl-*L*-tyrosine (CGlyT). If Equation 3 is applied to this case, one obtains Equations 4 and 5.

$$C_{CGlyP} = pE \cdot a \cdot b \cdot r_1 \quad (4)$$

$$C_{CGlyT} = pE \cdot a \cdot b \cdot r_2 \quad (5)$$

Thus,

$$\frac{C_{CGlyP}}{C_{CGlyT}} = \frac{r_1}{r_2} \quad (6)$$

Equation 6 implies two postulates that are accessible to experimental test. First, the numerical value of the proteolytic quotient C_{CGlyP}/C_{CGlyT}

TABLE I
Specificity of Carboxypeptidase

Temperature 25°.

Substrate	Enzyme concentration, protein N per cc test solution	pH	K*	C†	Quotient
	mg				
Carbobenzoxyglycyl- <i>L</i> -phenylalanine	0.00062	7.7	0.0053	8.5	1.8
Carbobenzoxyglycyl- <i>L</i> -tyrosine	0.00062	7.8	0.0029	4.7	
Carbobenzoxy- <i>L</i> -alanyl- <i>L</i> -phenylalanine	0.00062	7.8	0.0045	7.3	1.7
Carbobenzoxy- <i>L</i> -alanyl- <i>L</i> -tyrosine	0.00062	7.9	0.0026	4.2	
Carbobenzoxy- <i>L</i> -glutamyl- <i>L</i> -phenylalanine.	0.0031	7.9	0.0016	0.52	1.6
Carbobenzoxy- <i>L</i> -glutamyl- <i>L</i> -tyrosine	0.0031	7.8	0.0010	0.32	
Carbobenzoxy- <i>L</i> -phenylalanine	0.246	7.8	0.00027	0.0011	1.6
Carbobenzoxy- <i>L</i> -tyrosine	0.246	7.9	0.00017	0.00069	

$$* K = \frac{1}{\text{min.}} \log \frac{100}{100 - \% \text{ hydrolysis}}$$

$$† C = \frac{K}{\text{mg. protein N per cc. test solution}}$$

should depend only on the nature of the amino acid residues R_1 and R_2 , but should be independent of the nature of the A groups or B groups as long as either of these groups is identical for the two substrates that are compared. This postulate has been tested experimentally for pancreatic carboxypeptidase as the enzyme and for four pairs of substrates, containing one of the following A groups: carbobenzoxy, carbobenzoxyglycyl, carbobenzoxy-*L*-alanyl, and carbobenzoxy-*L*-glutamyl. As will be noted in Table I, the proteolytic quotient for each of the four pairs of substrates is the same within the limits of error of the experimental methods employed. Secondly, Equation 6 requires that the numerical value of the proteolytic quotient be independent of the individual nature of the carboxypeptidase;

i.e., it should be identical for the various carboxypeptidases. This is the result observed in the experiments reported earlier (1).

As was previously mentioned, the validity of Equation 3 may also be tested by following procedure (b); *i.e.*, by determining the proteolytic quotient for the action of an enzyme upon two substrates that differ only with respect to the *A* group. Then,

$$\frac{C_1}{C_2} = \frac{p_E \cdot a_1 \cdot b \cdot r}{p_E \cdot a_2 \cdot b \cdot r} = \frac{a_1}{a_2} \quad (7)$$

From the experimental data presented in Table I, it follows that the proteolytic quotient for the two substrates carbobenzoxyglycyl-*L*-phenylalanine (CGlyP) and carbobenzoxy-*L*-glutamyl-*L*-phenylalanine (CGluP) is

$$\frac{C_{\text{CGlyP}}}{C_{\text{CGluP}}} = \frac{8.5}{0.52} = 16.5 \quad (8)$$

For the substrates carbobenzoxyglycyl-*L*-tyrosine (CGlyT) and carbobenzoxy-*L*-glutamyl-*L*-tyrosine (CGluT), the following proteolytic quotient is obtained.

$$\frac{C_{\text{CGlyT}}}{C_{\text{CGluT}}} = \frac{4.7}{0.32} = 14.7 \quad (9)$$

The values obtained in Equations 8 and 9 agree within the experimental error of the method.

The values presented in Table I may also be utilized for a comparison of the action of pancreatic carboxypeptidase upon the pair of substrates CGluP and carbobenzoxy-*L*-phenylalanine (CP) and the pair CGluT and carbobenzoxy-*L*-tyrosine (CT). The following quotients are obtained.

$$\frac{C_{\text{CGluP}}}{C_{\text{CP}}} = 6600; \quad \frac{C_{\text{CGluT}}}{C_{\text{CT}}} = 6100$$

As postulated in Equation 7, the numerical value of the quotient for two substrates differing only with respect to the *A* groups is independent of the nature of the *R* groups, when these are identical in the two substrates compared. The data discussed in this communication are in accord with Equation 3 as are the previously reported data on several homospecific trypsinases and pepsinases (1, 2).

In the experiments with carboxypeptidases, trypsinases, and pepsinases, first order kinetics were obtained under the experimental conditions employed (1). Future experiments will show whether Equation 3 is obeyed in proteolytic systems in which the reaction kinetics are other than first order.

The authors wish to express their thanks to Miss Rosalind Joseph, Mr. Maurice Rapport, and Mr. Stephen M. Nagy for valuable assistance in this investigation.

EXPERIMENTAL

Carbobenzoxy-l-alanyl-l-phenylalanine

Carbobenzoxy-l-alanyl-l-phenylalanine Ethyl Ester—A dry ethereal solution of carbobenzoxy-l-alanyl chloride (from 8.9 gm. of carbobenzoxy-l-alanine) was added to an ethereal solution of l-phenylalanine ester (from 14 gm. of the hydrochloride). The reaction mixture was worked up as in the case of carbobenzoxyglycyl-l-phenylalanine ethyl ester (4). Yield, 12.9 gm. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 97–98°.

$C_{22}H_{24}O_4N_2$.	Calculated.	C 66.3, H 6.6, N 7.0
398.4	Found.	" 66.5, " 6.4, " 6.9

Carbobenzoxy-l-alanyl-l-phenylalanine—5 gm. of the above ester were dissolved in 50 cc. of methanol and 13 cc. of *N* NaOH were added. After 30 minutes, the solution was acidified with 14 cc. of *N* HCl. On removal of the methanol under reduced pressure, crystals were obtained. Yield, 4.5 gm. After recrystallization from methanol-water, the substance melted at 56–58°.

$C_{22}H_{22}O_4N_2$.	Calculated.	C 64.8, H 6.0, N 7.8
370.4	Found.	" 64.8, " 6.2, " 7.6

Carbobenzoxy-l-alanyl-l-tyrosine

Carbobenzoxy-l-alanyl-l-tyrosine Ethyl Ester—A dry ethereal solution of carbobenzoxy-l-alanyl chloride (from 4.5 gm. of carbobenzoxy-l-alanine) was added to an ethyl acetate solution of tyrosine ethyl ester (from 10 gm. of the hydrochloride). The reaction mixture was worked up as in the case of the phenylalanine analogue. Yield, 5.3 gm. After recrystallization from ethyl acetate-petroleum ether the substance melted at 138–139°.

$C_{22}H_{24}O_6N_2$.	Calculated.	C 63.8, H 6.3, N 6.8
414.4	Found.	" 63.7, " 6.3, " 6.8

Carbobenzoxy-l-alanyl-l-tyrosine—1.5 gm. of the above ester were dissolved in 20 cc. of methanol and 7.2 cc. of *N* NaOH were added. After 30 minutes, 7.5 cc. of *N* HCl were added. The methanol was evaporated off under reduced pressure and the resulting syrup taken up in ethyl acetate. The ethyl acetate extract was dried and on addition of petroleum ether a syrup was obtained which crystallized on standing in the cold. Yield, 1.0 gm. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 149–150°.

$C_{20}H_{22}O_6N_2$.	Calculated.	C 62.2, H 5.9, N 7.2
386.4	Found.	" 62.2, " 5.8, " 7.2

Enzymatic Studies

The crystalline carboxypeptidase was prepared and recrystallized according to the directions of Anson (5). The procedure employed in the determination of enzymatic hydrolysis was the same as that described earlier (4).

BIBLIOGRAPHY

1. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **141**, 763 (1941).
2. Bergmann, M., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **2**, 49 (1942).
3. Irving, G. W., Jr., Fruton, J. S., and Bergmann, M., *J. Biol. Chem.*, **138**, 231 (1941).
4. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134**, 225 (1940).
5. Anson, M. L., *J. Gen. Physiol.*, **20**, 663 (1937).

THE MULTIPLE SPECIFICITY OF CHYMOTRYPSIN

By JOSEPH S. FRUTON AND MAX BERGMANN

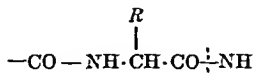
(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

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Previous experiments have shown that crystalline chymotrypsin hydrolyzes simple substrates at peptide linkages that involve the carbonyl group of an aromatic amino acid. Thus, benzoyl-*l*-tyrosylglycinamide is split to benzoyl-*l*-tyrosine and glycineamide (1). Since the point of attack is at an interior peptide bond, the enzyme has been classified as endopeptidase or proteinase (2). It is characteristic of this class of enzymes that they attack only such substrates as contain at least two peptide bonds in close proximity to each other (3).

In the experiments reported in the present communication, several new substrates for chymotrypsin have been employed for the further study of the specificity of this enzyme. Thus, twice crystallized chymotrypsin hydrolyzes glycyl-*l*-tyrosinamide (GTA) and glycyl-*l*-phenylalaninamide (GPA). The ready solubility of these substrates has permitted the determination of first order reaction velocity constants at several enzyme concentrations (Table I). Proteolytic coefficients of $C_{GTA} = 0.0065$ and $C_{GPA} = 0.0022$ were found at 25° and pH 7.8.

The hydrolysis of GTA and GPA occurs at the peptide linkages involving the carbonyl group of the aromatic amino acid residue. No free amino acid can be found in the hydrolysates by means of the Van Slyke ninhydrin method (Table II). Furthermore, following carbobenzoxylation of the hydrolysates, carbobenzoxyglycyl-*l*-tyrosine and carbobenzoxyglycyl-*l*-phenylalanine were isolated. The site of hydrolysis of GTA and GPA indicates that chymotrypsin endopeptidase requires the following arrangement of groups in the back-bone of its substrate.



where *R* is the side chain of tyrosine or phenylalanine. Chymotrypsin endopeptidase may therefore be classified as a carbonylproteinase (3).

It has now been observed that chymotrypsin slowly hydrolyzes *l*-tyrosinamide (TA) and *l*-phenylalaninamide (PA). The proteolytic coefficients for these two substrates were nearly identical; *i.e.*, 0.0003 (Table I). It is obvious that the enzymatic splitting of tyrosinamide and of phenylalaninamide cannot be attributed to an endopeptidase and that they

represent typical aminopeptidase actions. It must therefore be concluded that crystalline chymotrypsin shows two distinct specificities. One speci-

TABLE I

Kinetics of Chymotrypsin Action

Twice crystallized chymotrypsin, pH 7.8, 25°.

Substrate	Protein N per cc. test solution	K (first order)*	C†
	mg.		
Glycyl- <i>l</i> -tyrosinamide.....	0.14	0.0009	0.0064
	0.23	0.0015	0.0065
	0.32	0.0021	0.0066
	0.41	0.0027	0.0066
Glycyl- <i>l</i> -phenylalaninamide.....	0.29	0.0006	0.0021
	0.58	0.0013	0.0022
	0.87	0.0018	0.0021
	1.16	0.0027	0.0023
<i>l</i> -Tyrosinamide.. .. .	1.12	0.00032	0.00029
	1.68	0.00054	0.00032
<i>l</i> -Phenylalaninamide.....	1.12	0.00035	0.00031
	1.68	0.00054	0.00032

$$* K = \frac{1}{\text{min.}} \log \frac{100}{100 - \% \text{ hydrolysis}}$$

$$\dagger C = \frac{K}{\text{mg. protein N per cc. test solution}}$$

TABLE II

Comparison of Titration and Ninhydrin Data in Hydrolysis of Several Substrates by Chymotrypsin

Temperature 25°, pH 7.6 to 7.8.

Substrate	Protein N per cc. test solution	Time	Hydrolysis	
			Titration method	Ninhydrin method
	mg.	hrs.	per cent	per cent
Glycyl- <i>l</i> -tyrosinamide.. .. .	0.56	3	79	0
Glycyl- <i>l</i> -phenylalaninamide.	1.12	6	66	1
<i>l</i> -Tyrosinamide	1.12	20	53	48
<i>l</i> -Phenylalaninamide.....	1.12	20	52	49
<i>l</i> -Tyrosylglycinamide.....	1.12	24	65	44
<i>l</i> -Phenylalanylglycinamide....	1.12	24	60	
Glycylglycinamide.....	1.16	24	20	3
<i>l</i> -Tyrosyl- <i>l</i> -tyrosinamide	1.10	22	71	
<i>l</i> -Phenylalanyl- <i>l</i> -phenylalaninamide	1.10	22	34	
<i>l</i> -Phenylalanyl- <i>l</i> -tyrosinamide..	1.10	22	41	

ficity is that of a carbonylproteinase and the other is that of an aminopeptidase.

The presence of two different specificities in chymotrypsin is indicated also if the data in Table I are approached from a somewhat different point of view. In a previous paper of this series (4), it was shown that when an enzyme acts upon two substrates which differ only with respect to their amino acid residues R_1 and R_2 which bear the essential side chains (2), the following relation holds,

$$C_1/C_2 = r_1/r_2 \quad (1)$$

where C_1 and C_2 are the proteolytic coefficients for the hydrolysis of the two substrates, while r_1 and r_2 are numerical factors which depend on the nature of R_1 and R_2 . Equation 1 requires that the proteolytic quotient C_1/C_2 should depend only on the nature of R_1 and R_2 but should be independent of the structure of the remainder of the substrate molecule if the two substrates differ only with respect to the nature of R .

In the case of the hydrolysis of the substrates GTA, GPA, TA, and PA by chymotrypsin, Equation 1 has the form given in Equations 2 and 3,

$$C_{GTA}/C_{GPA} = r_T/r_P \quad (2)$$

$$C_{TA}/C_{PA} = r'_T/r'_P \quad (3)$$

where r_T is the numerical value that depends on the tyrosine residue in GTA and r_P is the numerical value that depends on the phenylalanine residue in GPA, while r'_T depends on the tyrosine residue in TA and r'_P depends on the phenylalanine residue in PA.

If the enzymatic actions upon which Equations 2 and 3 are based are the expression of one and the same enzymatic specificity of chymotrypsin, then the values for r_T/r_P and r'_T/r'_P should be identical and, consequently, the proteolytic quotients C_{GTA}/C_{GPA} and C_{TA}/C_{PA} should also be identical. It may be calculated easily from the values for C given in Table I that C_{GTA}/C_{GPA} equals 3 and C_{TA}/C_{PA} equals 1. It follows therefore that chymotrypsin hydrolyzes the substrates GTA and GPA with an enzymatic specificity different from that characteristic of the hydrolysis of TA and PA.

In a previous paper (3), chymotrypsin was classified as an imidoproteinase, because the enzyme liberates tyrosine from *l*-tyrosylglycinamide (TGA). This hydrolysis may now be attributed to the aminopeptidase activity of chymotrypsin.

The liberation of tyrosine is not the only action which chymotrypsin exerts upon TGA. Since chymotrypsin liberates from TGA a quantity of titratable carboxyl groups and since only a part of these carboxyls belongs to a free amino acid,¹ it must be concluded that the enzyme attacks both peptide bonds of the substrate, thus producing tyrosylglycine plus ammonia in addition to tyrosine plus glycineamide.

It was attempted to decide whether chymotrypsin must be regarded as a

¹ As determined by the ninhydrin method

mixture of two separate enzymes, one with proteinase activity and the other with aminopeptidase activity. Consequently, the ratio of these two activities in various chymotrypsin preparations was determined and efforts were made to alter this ratio by various procedures. These efforts, however, have so far been unsuccessful.

The twice crystallized chymotrypsin preparation employed in these experiments gave for the quotient C_{GPA}/C_{PA} a value of 7.4. After two more recrystallizations, the quotient remained unaltered (Table III). Furthermore, a preparation of γ -chymotrypsin, placed at our disposal by Dr. Kunitz, gave for C_{GPA}/C_{PA} a value of 7.3. Partial inactivation of chymotrypsin by alkali produced the same decrease in the activity toward GPA and PA. A solution of chymotrypsin was kept at pH 9.2 for 18 hours at

TABLE III

Action of Various Chymotrypsin Preparations on Glycyl-L-phenylalaninamide and L-Phenylalaninamide

Temperature 25°, pH 7.7 to 7.8.

Enzyme preparation	Protein N per cc. test solution	K		$\frac{C_{GPA}}{C_{PA}}$
		Glycyl- phenyl- alaninamide	Phenyl- alaninamide	
2 times crystallized chymotrypsin	mg.			
4 " " "	1.12	0.0026	0.00035	7.4
" " " "	1.20	0.0028	0.00038	7.4
γ -Chymotrypsin	0.88	0.0022	0.00030	7.3
Chymotrypsinogen	0.63	0.0000	0.0000	
Chymotrypsin from above chymotrypsinogen	0.63	0.0015	0.00020	7.5

25° with an attendant loss of 36 per cent in activity toward GPA and a loss of 40 per cent in activity toward PA.

A solution of chymotrypsinogen was found to be without activity on GPA and PA. On activation with a trace of crystalline trypsin, the resulting chymotrypsin split both of these substrates and the proteolytic quotient C_{GPA}/C_{PA} was 7.5 (Table III).

In the light of these results it must be regarded as probable that chymotrypsin is a protein which exhibits more than one, *i.e.* at least two, enzymatic specificities. The same appears to hold for γ -chymotrypsin. In this connection it should be recalled that the careful solubility studies of Kunitz (5) and Butler (6) have shown that chymotrypsinogen, α -chymotrypsin, and γ -chymotrypsin conform to the phase rule criteria of a pure protein.

It was mentioned above that tyrosylglycinamide is split slowly at the linkage involving the glycine carbonyl. Glycylglycinamide is also split at

a slow rate; an increase in titratable carboxyl groups was observed but the appearance of appreciable free amino acid could not be demonstrated in the hydrolysate (Table II). The question may be raised whether the splitting of glycylglycinamide and of glycyltyrosinamide can be attributed to a single chymotrypsin endopeptidase or whether there might be a third enzymatic specificity present in chymotrypsin. We are at present unable to perform the experiments necessary to decide this question.

TABLE IV

Action of Chymotrypsin on Acyl Peptide Amides

Enzyme concentration, 1.2 mg. of protein N per cc. of test solution. Temperature 25°, pH 7.1 to 7.5.

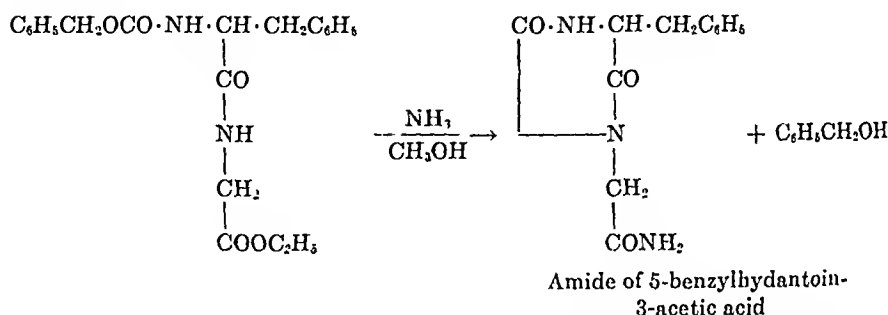
Substrate	Time	Hydrolysis
	hrs.	per cent
Carbobenzoxyl- <i>l</i> -tyrosylglycinamide (1)*	4.5	51
	24	89
Carbobenzoxyl- <i>l</i> -phenylalanylglycinamide	5	14
	24	32
Carbobenzoxylglycyl- <i>l</i> -tyrosylglycinamide (1)*	3	52
	6	75
Carbobenzoxylglycyl- <i>l</i> -phenylalanylglycinamide (1)*	3.5	11
	24	43
Carbobenzoxyl- <i>l</i> -tyrosyl- <i>l</i> -phenylalaninamide	24	10
Carbobenzoxyl- <i>l</i> -tyrosyl- <i>l</i> -tyrosinamide	24	18
Carbobenzoxyl- <i>l</i> -phenylalanyl- <i>l</i> -tyrosinamide	24	9
Carbobenzoxyl- <i>l</i> -phenylalanyl- <i>l</i> -phenylalaninamide	24	5
Carbobenzoxylglycyl- <i>l</i> -tyrosinamide	24	15
Carbobenzoxylglycyl- <i>l</i> -phenylalaninamide	24	4
Carbobenzoxyl- <i>l</i> -tyrosinamide	48	0
Carbobenzoxyl- <i>l</i> -phenylalaninamide	48	-1
Carbobenzoxyl- <i>l</i> -tyrosylglycine (1)*	48	0
Carbobenzoxyl- <i>l</i> -phenylalanylglycine	48	2

* Bibliographic reference.

Dipeptide amides such as *l*-tyrosyl-*l*-tyrosinamide, *l*-phenylalanyl-*l*-tyrosinamide, and *l*-phenylalanyl-*l*-phenylalaninamide were also found to be split by chymotrypsin (Table II). These compounds are sparingly soluble at pH 7 to 8 and consequently were not suitable for studies of reaction kinetics. The action of chymotrypsin on a number of other compounds was tested (Table IV). These included a series of acyl peptide amides that are difficultly soluble at pH 7 to 8.

In attempting to prepare carbobenzoxyl-*l*-phenylalanylglycinamide, we observed that, when carbobenzoxyl-*l*-phenylalanylglycine ethyl ester is treated with dry ammonia in methanol, benzyl alcohol is split out and a

substituted hydantoin is obtained. When the amide is boiled with 10 per cent sulfuric acid, the corresponding acidie compound is obtained. The melting points of the acid and amide correspond to those found by Gräna-cher and Landolt (7) who prepared these compounds by reduction of 5-benzalhydantoinacetic acid ethyl ester with subsequent saponification or amidation.



It is of interest that hydantoin formation was not found to occur in the treatment of several other carbobenzoxy peptide esters with ammonia, as described in the experimental section of this paper.

The authors wish to express their thanks to Mr. Maurice Rapport and Mr. Stephen M. Nagy for valuable assistance in this investigation.

EXPERIMENTAL

Glycyl-l-tyrosinamide Acetate

Carbobenzoxycyl-l-tyrosinamide—A solution of 7.8 gm. of carboben-zoxycyl-l-tyrosine ethyl ester (1) in 75 cc. of ammoniacal methanol was left at room temperature for 2 days. Concentration under reduced pressure yielded a syrup which was crystallized from ethanol-water. Yield, 5.3 gm.; m.p. 170°.

$\text{C}_{19}\text{H}_{21}\text{O}_5\text{N}_3$	Calculated	C 61.4, H 5.7, N 11.3
371.2	Found	" 61.5, " 5.7, " 11.3

Glycyl-l-tyrosinamide Acetate—1.85 gm. of the carbobenzoxy compound were hydrogenated in methanol solution in the presence of 0.3 cc. of glacial acetic acid. The filtrate was concentrated, yielding 1.4 gm. of the crystal-line product. The material was recrystallized from methanol-ethyl acetate.

$\text{C}_{11}\text{H}_{13}\text{O}_3\text{N}_3$	$\text{C}_7\text{H}_4\text{O}_2$	Calculated	C 52.5, H 6.4, N 14.1
297.2		Found	" 52.3, " 6.6, " 14.2
$[\alpha]_D^{25} = +28.0^\circ$ (10% in water)			

297 mg. of this substance were hydrolyzed by chymotrypsin to 98 per cent. The hydrolysate was concentrated to a small volume. 0.2 cc. of carbo-

benzoxy chloride and 2 cc. of N NaOH were added with cooling and shaking. The reaction mixture was acidified and extracted with ethyl acetate. The ethyl acetate layer was extracted with bicarbonate. When the bicarbonate layer was acidified, a syrup was obtained which crystallized on standing in the cold. Yield, 120 mg. After recrystallization from ethyl acetate, the melting point was 108° . The mixed melting point with an authentic sample of carbobenzoxyglycyl- l -tyrosine was 107 – 108° .

$C_{11}H_{10}O_4N_2$.	Calculated.	C 61.3, H 5.4, N 7.5
372.2	Found.	" 61.2, " 5.6, " 7.7

Glycyl- l -phenylalaninamide Acetate

Carbobenzoxyglycyl- l -phenylalaninamide—The syrupy ester (prepared from 3.8 gm. of carbobenzoxyglycyl chloride and 4.5 gm. of phenylalanine ethyl ester hydrochloride in the manner described earlier (8)) was dissolved in 50 cc. of methanol previously saturated with ammonia at 0° . After 2 days the solution was concentrated and the resulting syrup was crystallized from methanol-water. The material was recrystallized from methanol-water. Yield, 4.8 gm.; m.p. 130° .

$C_{11}H_{11}O_4N_2$.	Calculated.	C 64.3, H 6.0, N 11.8
355.4	Found.	" 64.3, " 6.0, " 11.9

Glycyl- l -phenylalaninamide Acetate—1.8 gm. of the carbobenzoxy compound were hydrogenated in methanol solution in the presence of 0.3 cc. of glacial acetic acid. The filtrate was concentrated, yielding 1.2 gm. of the crystalline product. The material was recrystallized from methanol-ethyl acetate.

$C_{11}H_{13}O_2N_2 \cdot C_2H_4O_2$.	Calculated.	C 55.5, H 6.8, N 14.9
281.2	Found.	" 55.6, " 6.7, " 14.8
$[\alpha]_D^{25} = +23.8^{\circ}$ (5% in water)		

281 mg. of this substance were hydrolyzed by chymotrypsin to 95 per cent. The reaction mixture was worked up as in the case of glycyltyrosinamide. The yield of crude carbobenzoxy derivative was 135 mg. After recrystallization from ethyl acetate-ether, the melting point was 125° . The mixed melting point with an authentic sample of carbobenzoxyglycyl- l -phenylalanine was 125 – 126° .

$C_{11}H_{13}O_2N_2$.	Calculated.	C 64.0, H 5.6, N 7.8
356.4	Found.	" 64.1, " 5.8, " 8.0

l -Tyrosinamide Acetate

This material was prepared as described previously (9). 240 mg. of this substance were hydrolyzed by chymotrypsin. After 48 hours at 25° the characteristic crystals (73 mg.) of tyrosine were obtained. Calculated for tyrosine, 7.7 per cent NH_2-N ; found, 7.6 per cent NH_2-N .

l-Phenylalaninamide Acetate

Carbobenzoxy-l-phenylalaninamide—8 gm. of carbobenzoxy chloride and 4 gm. of MgO were added to an ethyl acetate solution of phenylalanine ethyl ester (prepared from 10 gm. of the hydrochloride). The excess chloride was destroyed with pyridine and the ethyl acetate layer was washed with dilute hydrochloric acid, water, dilute bicarbonate, and water. The dried ethyl acetate solution was concentrated to yield a syrup which was dissolved in 50 cc. of methanol previously saturated with ammonia at 0°. After 2 days at room temperature, the solution was concentrated to give 6.5 gm. of the crystalline material. M.p. 167°.

$C_{17}H_{18}O_3N_2$.	Calculated.	C 68.4, H 6.1, N 9.4
298.2	Found.	" 68.2, " 6.2, " 9.3

l-Phenylalaninamide Acetate—1.5 gm. of the above carbobenzoxy compound were hydrogenated in methanol solution in the presence of 0.35 cc. of glacial acetic acid. The filtrate was concentrated to a syrup which was crystallized on treatment with ethyl acetate. Yield, 0.9 gm. After recrystallization from ethyl acetate, the substance melted at 119–120°.

$C_9H_{12}ON_2 \cdot C_2H_4O_2$.	Calculated.	C 58.9, H 7.2, N 12.5
224.2	Found.	" 59.2, " 7.4, " 12.4

l-Tyrosylglycinamide Acetate

1.4 gm. of carbobenzoxy-*l*-tyrosylglycinamide (1) were hydrogenated in methanol containing 0.3 cc. of glacial acetic acid. The filtrate was concentrated to a syrup which was crystallized by careful addition of ethyl acetate. Yield, 1.0 gm. The material was recrystallized from methanol-ethyl acetate.

$C_{11}H_{16}O_3N_3 \cdot C_2H_4O_2$.	Calculated.	C 52.5, H 6.4, N 14.1
297.2	Found.	" 52.5, " 6.2, " 14.2
$[\alpha]_D^{25} = +70.9^\circ$ (6% in water)		

149 mg. of this compound were hydrolyzed by chymotrypsin. After 47 hours the hydrolysis attained 80 per cent splitting of one peptide linkage. The enzyme was coagulated by heating to 100° and the filtrate was concentrated to 4 cc. On cooling, the characteristic crystals (32 mg.) of tyrosine were obtained. The material was recrystallized from hot water. Calculated for tyrosine, 7.7 per cent NH_2-N ; found, 7.7 per cent NH_2-N .

l-Phenylalanylglycinamide Acetate

Carbobenzoxy-l-phenylalanylglycinamide—3 gm. of carbobenzoxy-*l*-phenylalanyl chloride (10) were added to an ethyl acetate solution of glycine (prepared in the usual manner from 10 gm. of glycine hydro-

chloride). After 30 minutes the reaction mixture was filtered. The ethyl acetate solution was washed successively with water, dilute hydrochloric acid, water, dilute bicarbonate, and water. After drying, the ethyl acetate solution was concentrated *in vacuo*. Addition of petroleum ether yielded 2.9 gm. of the product. The substance was recrystallized from methanol-water. M.p. 134°.

$C_{11}H_{21}O_4N_3$.	Calculated.	C 64.3, H 6.0, N 11.8
355.4	Found.	" 64.0, " 6.2, " 11.6

l-Phenylalanylglycinamide Acetate—1.6 gm. of the above carbobenzoxy compound were hydrogenated in methanol containing 0.4 cc. of glacial acetic acid. On evaporation of the filtrate, crystals (1.1 gm.) were obtained. The substance was recrystallized from methanol-ether.

$C_{11}H_{11}O_4N_3 \cdot C_2H_4O_2$.	Calculated.	C 55.5, H 6.8, N 14.9
281.2	Found.	" 55.6, " 7.0, " 14.7
$[\alpha]_D^{25} = +68.2^\circ$ (5% in water)		

l-Tyrosyl-*l*-tyrosinamide Acetate

Carbobenzoxy-l-tyrosyl-l-tyrosinamide—2 gm. of the O-acetyl ethyl ester (11) were treated with 40 cc. of methanol previously saturated with ammonia at 0°. The reaction mixture stood at room temperature for 2 days and was then concentrated under reduced pressure. Addition of ether-petroleum ether yielded 1.4 gm. of the material. M.p. 187–189°.

$C_{21}H_{27}O_6N_3$.	Calculated.	C 65.4, H 5.7, N 8.8
477.6	Found.	" 65.2, " 5.8, " 8.8

l-Tyrosyl-*l*-tyrosinamide Acetate—1 gm. of the carbobenzoxy compound was hydrogenated catalytically in methanol in the presence of 0.15 cc. of glacial acetic acid. The filtrate was concentrated and the product was crystallized by the addition of ethyl acetate. Yield, 0.7 gm.

$C_{18}H_{20}O_4N_3 \cdot C_2H_4O_2$.	Calculated.	C 59.8, H 6.0, N 10.4
402.4	Found.	" 60.0, " 5.9, " 10.3

Carbobenzoxy-l-tyrosyl-l-phenylalaninamide

N-Carbobenzoxy-*O*-acetyl-*l*-tyrosyl-*l*-phenylalanine Ethyl Ester—4.0 gm. of *N*-carbobenzoxy-*O*-acetyl-*l*-tyrosyl chloride (11) were added to an ethyl acetate solution of *l*-phenylalanine ethyl ester (prepared from 7 gm. of the hydrochloride). 1 hour later the mixture was filtered and the filtrate was washed successively with water, dilute HCl, water, dilute bicarbonate, and water. After drying over Na_2SO_4 , the solution was concentrated and petroleum ether was added carefully. 3.6 gm. of a crystalline precipitate

were obtained. After recrystallization from ethyl acetate-ether, the melting point was 170°.

$C_{30}H_{32}O_7N_2$.	Calculated.	C 67.7, H 6.1, N 5.3
532.6	Found.	" 67.6, " 6.3, " 5.3

Carbobenzoxyl-l-tyrosyl-l-phenylalaninamide—2 gm. of the above ester were treated with 40 cc. of methanol previously saturated with dry ammonia at 0°. The reaction mixture was left at room temperature for 2 days. Crystals were obtained on concentrating the solution. Yield, 1.6 gm.; m.p. 220°.

$C_{26}H_{27}O_6N_2$.	Calculated.	C 67.6, H 5.9, N 9.1
461.6	Found.	" 67.6, " 6.2, " 8.9

Attempts to prepare the corresponding tyrosylphenylalaninamide acetate in crystalline form have been unsuccessful to date.

l-Phenylalanyl-l-tyrosinamide

Carbobenzoxyl-l-phenylalanyl-l-tyrosine Ethyl Ester—4.2 gm. of carbobenzoxylphenylalanyl chloride were added to an ethyl acetate solution of tyrosine ethyl ester (prepared from 10 gm. of the hydrochloride). After 30 minutes the mixture was filtered and the filtrate was washed in the usual manner. The dry ethyl acetate solution was concentrated to a small volume and the product was crystallized by the addition of petroleum ether. Yield, 3.0 gm.; m.p. 162°.

$C_{28}H_{30}O_6N_2$.	Calculated.	C 68.6, H 6.2, N 5.7
490.5	Found.	" 68.8, " 6.3, " 5.6

Carbobenzoxyl-l-phenylalanyl-l-tyrosinamide—1.5 gm. of the above ester were dissolved in 50 cc. of methanol previously saturated with ammonia at 0°. After standing 2 days at room temperature, the solution was concentrated, yielding 1.0 gm. of the material. M.p. 221°.

$C_{26}H_{27}O_6N_2$.	Calculated.	C 67.6, H 5.9, N 9.1
461.5	Found.	" 67.6, " 6.0, " 9.1

l-Phenylalanyl-l-tyrosinamide—1 gm. of the carbobenzoxyl compound was hydrogenated in methanol in the presence of 0.15 cc. of glacial acetic acid. The filtrate was evaporated down, yielding a syrup which was dissolved in hot water. The addition of N NaOH to pH 7 gave a crystalline precipitate which was recrystallized from hot water. Yield, 0.4 gm.; m.p. 180°.

$C_{18}H_{21}O_3N_2$.	Calculated.	C 66.0, H 6.5, N 12.8
327.4	Found.	" 65.7, " 6.6, " 12.7

l-Phenylalanyl-*l*-phenylalaninamide

Carbobenzoxyl-l-phenylalanyl-l-phenylalanine Ethyl Ester—4.2 gm. of carbobenzoxyl-*l*-phenylalanyl chloride were added to an ethyl acetate solution of *l*-phenylalanine ethyl ester (prepared from 10 gm. of the hydrochloride). The mixture was worked up in the manner employed in the coupling with tyrosine ethyl ester. Yield, 4.3 gm.; m.p. 140°.

$C_{21}H_{16}O_2N_2$	Calculated.	C 70.7, H 6.4, N 5.9
474.5	Found.	" 70.9, " 6.4, " 5.9

Carbobenzoxyl-l-phenylalanyl-l-phenylalaninamide—1.6 gm. of the above ester were dissolved in 50 cc. of methanol previously saturated with ammonia at 0°. The amide crystallized out on standing at room temperature for 24 hours. Yield, 1.3 gm.; m.p. 230°.

$C_{21}H_{17}O_2N_2$	Calculated.	C 70.1, H 6.1, N 9.4
445.5	Found.	" 70.1, " 6.0, " 9.4

l-Phenylalanyl-*l*-phenylalaninamide—1 gm. of the carbobenzoxyl compound was suspended in methanol and hydrogenated catalytically in the presence of 0.16 cc. of glacial acetic acid. At the end of the hydrogenation the carbobenzoxyl compound had gone into solution. The filtrate was concentrated to a syrup which was dissolved in hot water. The addition of *N* NaOH to pH 7 gave a crystalline precipitate which was recrystallized from hot water. Yield, 0.7 gm.; m.p. 138°.

$C_{18}H_{17}O_2N_2$	Calculated.	C 69.4, H 6.8, N 13.5
311.4	Found.	" 69.5, " 6.8, " 13.3

Glycylglycinamide Acetate

Carbobenzoxylglycylglycinamide—6 gm. of carbobenzoxylglycylglycine ethyl ester² (m.p. 82–83°) were dissolved in 50 cc. of methanol previously saturated with dry ammonia at 0°. After 2 days at room temperature, the amide had separated out. Yield, 4.9 gm. After recrystallization from methanol the substance melted at 179–181°.

$C_{12}H_{13}O_2N_2$	Calculated.	C 54.3, H 5.7, N 15.8
265.2	Found.	" 54.0, " 5.5, " 15.7

Glycylglycinamide Acetate—3 gm. of the above amide were hydrogenated in methanol containing 0.65 cc. of glacial acetic acid. On concentration of the filtrate, the product separated out. Yield, 1.9 gm. The substance was recrystallized from methanol-ethyl acetate.

$C_7H_9O_2N_2 \cdot C_2H_3O_2$	Calculated.	C 37.7, H 6.9, N 22.0
191.2	Found.	" 37.8, " 7.0, " 22.0

² This compound was prepared previously by Rinke (12).

Carbobenzoxy-l-phenylalanylglycine—1.2 gm. of carbobenzoxyphenylalanylglycine ethyl ester (1) were dissolved in 25 cc. of methanol and 3.2 cc. of N NaOH were added. After 20 minutes the solution was acidified with 3.5 cc. of N HCl and the methanol was evaporated off. The resulting crystals were recrystallized from hot water. Yield, 0.8 gm.; m.p. 152°.

$C_{19}H_{20}O_6N_2$.	Calculated.	C 64.0, H 5.7, N 7.9
356.4	Found.	" 63.8, " 5.8, " 7.8

Amide of 5-Benzylhydantoin-3-acetic Acid—4.4 gm. of carbobenzoxy-l-phenylalanylglycine ethyl ester were dissolved in 50 cc. of methanol previously saturated with ammonia at 0°. After 2 days at room temperature, the solution was concentrated to yield a crystalline residue. Yield, 2.4 gm. After recrystallization from hot water the substance melted at 218°. Gränacher and Landolt (7) report a melting point of 216–218° for this compound.

$C_{12}H_{13}O_3N_3$.	Calculated.	C 58.2, H 5.3, N 17.0
247.2	Found.	" 57.9, " 5.2, " 16.9

5-Benzylhydantoin-3-acetic Acid—0.5 gm. of the above compound was heated under a reflux with 20 cc. of 10 per cent sulfuric acid for 3 hours. On cooling, crystals separated. Yield, 0.4 gm. After recrystallization from hot water, the substance melted at 185–186°. Gränacher and Landolt (7) report a melting point of 181–183° for this compound.

$C_{12}H_{12}O_4N_2$.	Calculated.	C 58.0, H 4.9, N 11.3
248.2	Found.	" 57.9, " 4.8, " 11.1

Enzymatic Studies

The crystalline chymotrypsin and chymotrypsinogen were prepared and recrystallized according to the directions of Kunitz and Northrop (13). The substrate concentration was 0.05 mm per cc. in the case of the soluble compounds. 0.125 mm of the insoluble substrates was weighed into 2.5 cc. volumetric flasks in which the enzymatic hydrolysis was performed. The liberated carboxyl groups were measured by the microtitration method of Grassmann and Heyde (14). With this method, more precise values are obtained with glycyl-l-phenylalaninamide than with the corresponding tyrosine compound. Glycyl-l-phenylalaninamide is to be recommended, therefore, for quantitative estimation of chymotrypsin activity. The ninhydrin method for the determination of free amino acids was applied as described by Van Slyke and his collaborators (15).

BIBLIOGRAPHY

1. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **118**, 405 (1937).
2. Bergmann, M., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **2**, 49 (1942).

3. Fruton, J. S., Irving, G. W., Jr., and Bergmann, M., *J. Biol. Chem.*, **141**, 763 (1941).
4. Bergmann, M., and Fruton, J. S., *J. Biol. Chem.*, **145**, 247 (1942).
5. Kunitz, M., *J. Gen. Physiol.*, **22**, 207 (1938).
6. Butler, J. A. V., *J. Gen. Physiol.*, **24**, 189 (1940).
7. Gränacher, C., and Landolt, H., *Helv. chim. acta*, **10**, 799 (1927).
8. Hofmann, K., and Bergmann, M., *J. Biol. Chem.*, **134**, 225 (1940).
9. Behrens, O. K., and Bergmann, M., *J. Biol. Chem.*, **129**, 587 (1939).
10. Bergmann, M., Zervas, L., Rinke, H., and Schleich, H., *Z. physiol. Chem.*, **224**, 33 (1934).
11. Bergmann, M., Zervas, L., Salzmann, L., and Schleich, H., *Z. physiol. Chem.*, **224**, 17 (1934).
12. Rinke, H., Thesis, Leipzig (1934).
13. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **19**, 991 (1936).
14. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
15. Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).

THE INFLUENCE OF LACTOSE AND ITS HYDROLYSIS PRODUCTS ON THE ABSORPTION OF CALCIUM

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The effect of lactose on the absorption and retention of calcium has been the subject of numerous investigations. Dragstedt and Peacock (1) showed that the onset of tetany in thyroparathyroidectomized dogs could be prevented by the oral administration of diets high in lactose. In similar experiments Inouye (2) confirmed these findings and noted that none of the other common sugars with the exception of galactose was effective. Since parenteral administration of lactose and galactose did not prevent tetany in these animals, it appeared that the site of action of lactose and galactose was in the alimentary tract. Inouye postulated that the metabolism of lactose was interrelated in some way with that of calcium, since the serum calcium levels of the experimental dogs which received lactose or galactose after the removal of the parathyroids did not drop as much as those of parathyroidectomized animals which had not received these sugars. Bergeim (3) reported that the absorption of calcium of rachitic rats was greater if lactose was fed at a 25 per cent level than if similar rats were fed other common sugars at the same level. The increased calcium absorption was ascribed to a higher intestinal acidity associated with lactic acid formation by bacterial enzymes. This phase of the subject has been adequately reviewed and subjected to further study by Robinson and his coworkers (4, 5). Their work has demonstrated that the influence of lactose on the absorption of calcium from the intestine is modified both by the composition of the diet to which the lactose has been added and the nature of the administered calcium salt. Robinson, Stewart, and Luckey (6) have studied the absorption of calcium, supplied as calcium lactate and calcium chloride, from Thiry-Vella loops in dogs and have demonstrated that although an acid pH (4.3 to 4.4) was favorable for the absorption of calcium administered as a solution of calcium chloride, the same pH was unfavorable for the absorption of calcium administered as a solution of calcium lactate. Their experiments have also shown that the pH of an acid or an alkaline solution is in most cases rather rapidly readjusted to a pH which is normal for the intestinal contents of that particular intestinal segment. It seems improbable from a consideration of these data that the favorable effect of lactose on calcium metabolism can be explained by the production of an

acid medium in the gut. There is nevertheless sufficient evidence that the inclusion of relatively high levels of lactose in the diet does increase the retention of calcium (7, 8). The mechanism, however, by which this effect of lactose is achieved is still obscure.

So far as the authors are aware, no attempt has been made to study the influence of lactose or its hydrolysis products upon the rate of disappearance of calcium from the intestinal tract of an intact animal by the determination of the residual calcium at a given interval following the administration of a definite quantity of calcium by stomach catheter (Cori technique). It is the purpose of these experiments to utilize this method to study the influence of lactose and its hydrolysis products, glucose and galactose, on calcium absorption.

EXPERIMENTAL

Young male rats were fed a low calcium diet (15 per cent casein, 58 per cent starch, 23 per cent Crisco, 2 per cent agar, and a 2 per cent calcium-free salt mixture¹) for a period of 5 to 13 days. This diet is essentially the same as that used by Adolph and Liang (9) in their investigation on the excretion of parenterally administered calcium into the gastrointestinal tract. After several days on such a diet the calcium content of the gastrointestinal tract is low and rather uniform in amount. In some experiments this diet was modified by the replacement of a portion of the starch by glucose or lactose. After the rats had been fed on the low calcium diet for at least 5 days, they were fasted for 24 hours previous to the administration of definite, analyzed quantities of calcium lactate by stomach tube. 3 hours later the animals were anesthetized with chloroform and the entire gastrointestinal tract was removed for analysis. After the material was dried in an oven at 90–100° for 24 hours, the residue was quantitatively transferred to platinum crucibles and ashed in a muffle furnace at 500–550°. The ash was dissolved in the minimum volume of 6 N hydrochloric acid and analyzed for calcium by the method of Wang (10). A series of thirteen rats weighing from 110 to 150 gm. was fed the calcium-free diet for a period of 5 days. After a 24 hour fast these rats were killed and the entire gastrointestinal tract was analyzed for calcium by the above procedure. The individual analyses ranged from 1.45 to 4.86 mg. of calcium per rat, but 80 per cent of these values was between 2 and 4 mg., with a mean value of 2.87 mg. of calcium. These blank values are somewhat higher than those presented by Adolph and Liang, since the calcium contained in the gut wall is included in the present values, while their determinations were made on intestinal washings. In subsequent calculations of the rate of absorption of calcium a

¹ Ca-free salt mixture (Adolph, W. H., Wang, C. H., and Smith, A. H., *J. Nutrition*, 16, 291 (1938)).

correction for this blank value of 2.87 mg. has been made. As presented in Table I, the absorption of calcium has been calculated both as mg. per rat per hour and as mg. per 100 gm. of rat per hour (absorption coefficient).

DISCUSSION

An inspection of Table I indicates that in general the rate of absorption of calcium increases progressively with the concentration of the adminis-

TABLE I

Calcium Absorption in Albino Rat Following Oral Administration of Calcium Lactate

The figures in parentheses are the values for the individual rats.

Group No	No of rats	Weight range	Calcium fed	Calcium contained in*	Calcium absorbed	
					Per rat per hr	Per 100 gm. rat per hr
		gm	mg.		mg	mg.
1	3	118-133	8.5	Water	1.14 (0.87-1.58)	0.92 (0.66-1.34)
2	5	112-129	9.4	"	1.26 (0.76-1.58)	1.06 (0.59-1.41)
3	4	132-150	15.1	5% lactose	2.81 (2.62-3.03)	2.05 (1.75-2.26)
4	4	101-130	15.5	Water	2.86 (2.42-3.23)	2.45 (1.98-2.62)
5	5	114-131	18.3	5% galactose	3.02 (2.75-3.73)	2.44 (2.10-3.27)
6	6	121-133	18.5	5% lactose	2.46 (1.18-3.05)	1.96 (0.91-2.40)
7	5	110-118	19.9	Water	3.53 (2.73-3.83)	3.09 (2.31-3.39)
8	5	109-117	19.9†	"	3.18 (2.80-3.59)	2.82 (2.54-3.29)
9	4	92-101	20.6	5% galactose	3.85 (3.11-4.41)	3.97 (3.38-4.37)
10	4	96-102	20.6	5% glucose	3.82 (3.36-4.56)	3.64 (3.46-3.94)
11	2	100-102	21.0	Water	3.65 (3.44-3.85)	3.61 (3.37-3.85)
12	6	89-101	23.0‡	"	3.62 (2.76-5.09)	3.94 (3.12-5.05)
13	3	77-93	23.0‡	"	4.38 (4.22-4.66)	5.06 (4.53-5.52)
14	6	117-126	26.5	20% lactose	2.96 (2.08-4.26)	2.43 (1.76-3.38)

* The calcium lactate was administered in every case in a volume of 2 cc. of fluid

† 20 per cent glucose replaced 20 per cent of the starch in the basal diet of the rats in Groups 8 and 13

‡ 20 per cent lactose replaced 20 per cent of the starch in the basal diet of the rats of Group 12

tered calcium lactate. If the average absorption in mg. of calcium per rat per hour for each group is plotted against the mg. of calcium fed, most of the points fall rather close to a line which represents the absorption of 1 mg. of calcium per hour for each 5.8 mg. of calcium fed. It is to be noted that the absorption of calcium in the rats of Groups 3, 5, 6, 9, and 10, which have received the calcium lactate in 5 per cent solutions of various sugars, is approximately the same as that in rats which have been given similar amounts of calcium lactate in water solution. This is well illustrated by Groups 3 and 4, which received 15.1 and 15.5 mg. of calcium, respectively.

The calcium absorption per hour for the former group, which had received the calcium lactate in 5 per cent lactose solution, was 2.81 mg., as compared to 2.86 mg. for the latter group, receiving essentially the same amount of calcium lactate in water solution. The rate of absorption of calcium for the rats of Group 6, which were given the calcium lactate in lactose solution, appears to be less than would be predicted for this level of calcium intake. This is due to the abnormally low calcium absorption of one rat (1.18 mg. of calcium per hour). If this value is discarded, the average calcium absorption per hour for the remaining five rats is 2.8 mg.

Since Nalder (11) has shown that lactose is rapidly hydrolyzed to glucose and galactose in the intestine of the rat, the effect of the presence of these sugars upon calcium absorption was included in the present study. Assuming that the average absorption is 1 mg. of calcium per hour for each 5.8 mg. of calcium fed, the rats of Group 5, which received 18.3 mg. of calcium in 5 per cent galactose, should have absorbed 3.26 mg. of calcium. This compares favorably with the average value of 3.02 mg. experimentally obtained for this series of animals. Calculated in the same manner the theoretical hourly absorption by the rats of Group 9 (calcium administered in 5 per cent galactose) and of Group 10 (calcium administered in 5 per cent glucose) should be 3.55 mg. The calcium absorptions recorded in Table I for these two groups of rats (3.85 and 3.82 mg. of calcium, respectively, per hour) are well within the experimental error of this type of procedure. Two rats in a control group (Group 11) which received a comparable amount of calcium lactate in water solution absorbed approximately the same amount of calcium per hour as the rats of Groups 9 and 10.

In the earlier experiments, previously cited (7, 8), in which lactose was shown to have a favorable influence on calcium retention, this sugar was usually present in relatively large amounts (25 to 40 per cent of the diet). In the present experiments, six rats (Group 14) were given 26.5 mg. of calcium as calcium lactate in 20 per cent lactose solution. The average calcium absorption for this group of rats is 2.96 mg. per hour, which is much less than expected for this calcium intake. Unfortunately there is no control group which received an equal amount of calcium lactate in water solution, but the rats in Groups 11, 12, and 13, receiving smaller amounts of calcium lactate in water solution, absorbed a greater amount of calcium. In any case, it may be definitely stated that the presence of lactose in higher concentration did not result in a more rapid absorption of calcium.

In order to determine whether the feeding of lactose over a longer period of time would alter conditions in the intestine, so that the absorption of calcium would be facilitated, one group of rats (Group 12) was fed for 10 days on a diet in which 20 per cent of the starch of the basal diet was re

placed by lactose. The calcium absorption for this group of rats was actually about 10 per cent lower than the amount which might have been expected on the basis of the amount administered. Two groups of rats (Groups 8 and 13) were fed a diet in which 20 per cent of the starch in the basal diet was replaced by glucose. The amount of calcium absorbed per hour after the administration of calcium lactate in water solution was slightly less than the calculated amount for the rats of Group 8, and slightly more than the calculated amount for the rats of Group 13.

Nordbø (12) has called attention to the fact that lactose forms an un-ionized calcium complex and suggested that by the formation of such a salt the risk of calcium precipitation in the intestine as the insoluble calcium phosphate is decreased. The present data, however, do not support the view that the presence of moderate amounts of lactose in the gut facilitates the absorption of calcium. 5 per cent solutions of the sugars were chosen for the present study, since this concentration is approximately that of lactose in milk.

SUMMARY

A simple direct method for the study of the rate of absorption of calcium from the gut of the albino rat is presented. The rate of absorption of the calcium administered orally as calcium lactate has been studied. Under the conditions of these experiments, the presence of lactose or its hydrolysis products, glucose and galactose, did not increase the rate of calcium absorption. In higher concentration, lactose apparently inhibited calcium absorption.

BIBLIOGRAPHY

- 1 Dragstedt, L. R., and Peacock, S. C., *Am J Physiol*, **64**, 424 (1923)
- 2 Inouye, T., *Am J Physiol*, **70**, 524 (1924)
- 3 Bergeim, O., *J Biol Chem*, **70**, 35 (1926)
- 4 Robinson, C. S., Huffman, C. F., and Mason, M. F., *J Biol Chem*, **84**, 257 (1929)
- 5 Robinson, C. S., and Duncan, C. W., *J Biol Chem*, **92**, 435 (1931)
- 6 Robinson, C. S., Stewart, D. E., and Luckey, F., *J Biol Chem*, **137**, 283 (1941)
- 7 Kline, O. L., Keenan, J. A., Elvehjem, C. A., and Hart, E. B., *J Biol Chem*, **98**, 121 (1932).
- 8 Outhouse, J., Smith, J., and Twomey, I., *J Nutrition*, **15**, 257 (1938)
- 9 Adolph, W. H., and Liang, C., *J Biol Chem*, **137**, 517 (1941)
- 10 Wang, C. C., *J Biol Chem*, **111**, 443 (1935)
- 11 Nalder, M. E., University microfilms, Publication No 224 (1940); *Microfilm Abst*, **3**, No 1, 3 (1941)
- 12 Nordbø, R., *J Biol. Chem*, **128**, 745 (1939)

MAMMALIAN TYROSINASE AND DOPA OXIDASE

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Most of the experimental work concerning the mechanism of melanin production in mammals indicates that the pigment is formed from the amino acid, dihydroxyphenylalanine (dopa), by the action of a specific oxidative enzyme. The evidence for this reaction has in large part been presented by Bloch (1), whose experiments were confirmed by Laidlaw and others (2). Bloch treated frozen sections of skin with a 1 per cent aqueous solution of dihydroxyphenylalanine buffered at pH 7.4, and over a period of 24 hours noted the appearance of a dark pigment in the cytoplasm of the melanoblasts. The reaction was inhibited by cyanide ion and by heat and failed to occur with a number of other substrates, including tyrosine. Bloch concluded that a specific enzyme, which he named dopa oxidase, is responsible for the formation of melanin.

Although several tyrosine-oxidizing enzymes have been extracted from certain plants and insects, there is no conclusive evidence for the existence of a mammalian tyrosinase. A number of attempts to demonstrate this enzyme in extracts of mammalian skin by means of a color reaction have been made, but the results are contradictory and inconclusive (3-9). Color reactions with dihydroxyphenylalanine have usually been obtained with such extracts, but the amount of dopa oxidase extractable is apparently very small. It seems evident, therefore, that normal pigmented tissues do not provide a rich enough source for any detailed study of this enzyme system.

The melanoma, a tumor composed chiefly of melanin-producing cells, might be expected to provide a richer source of the enzyme. Several experiments (10, 11) have indicated that extracts of melanomata possess activity against catechol derivatives, but the presence of tyrosinase has not been proved. The melanoma which we have utilized arose spontaneously in the skin of the ear of a chocolate-brown mouse and was reported by Harding and Passey in 1930 (12). It was found to be easily transplantable to mice of all colors, including albinos, and it has been carried for a number of years in the Rockefeller Institute strain of albino mice. Although slow growing, the neoplasm attains a relatively enormous size, often as much as one-third that of its host, in about 3 months. Grossly, it is a soft, encapsulated, jet-black nodule. Microscopically, it is made up of two cellular elements, one a cuboidal cell containing a relatively

small amount of pigment and comprising the bulk of the tumor, and the other a large cell which is loaded with pigment, seems to lie in the interstices, and is probably a macrophage.

EXPERIMENTAL

As a prospective source of melanin-producing enzymes, the transplantable melanoma is ideal, both because of its uniformity and because of the fact that it can be grown in theoretically unlimited quantity. It therefore seemed feasible to test its enzymatic activity. Accordingly, a tumor was removed, ground thoroughly with sand, and made into a thin brei with saline. The substrates *l*-phenylalanine, *l*-tyrosine, and *l*-dihydroxyphenylalanine were added to the brei in the Warburg apparatus and the oxygen up-

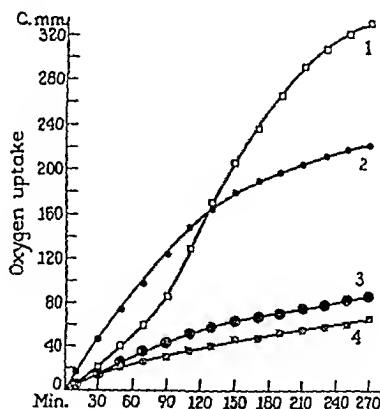


FIG. 1. The oxidation of tyrosine and dihydroxyphenylalanine by tumor brei. Each flask contained 2.5 cc. of tumor brei or the equivalent of 0.5 gm. of tumor. Curve 1, tyrosine 1.0 mg., pH 7.4; Curve 2, dihydroxyphenylalanine 1.0 mg., pH 6.5; Curve 3, phenylalanine 1.0 mg., control, pH 7.4; Curve 4, control, pH 6.5.

take measured. The temperature was maintained at 38°. When dihydroxyphenylalanine was the substrate, a phosphate buffer at pH 6.5 was used, because this amino acid undergoes appreciable autoxidation at physiological pH. With the remaining substrates, a buffer at pH 7.4 was employed.

As can be seen in Fig. 1, no oxidation of phenylalanine occurred. This has been the case in several other experiments lasting as long as 8 hours. Both tyrosine and dihydroxyphenylalanine underwent vigorous oxidation in the presence of the tumor brei. When the oxygen absorption ceased, the samples were removed and centrifuged. The supernatant fluids from the phenylalanine and control samples were light tan in color; those from the tyrosine and dihydroxyphenylalanine samples were quite black, indicating that melanin was produced.

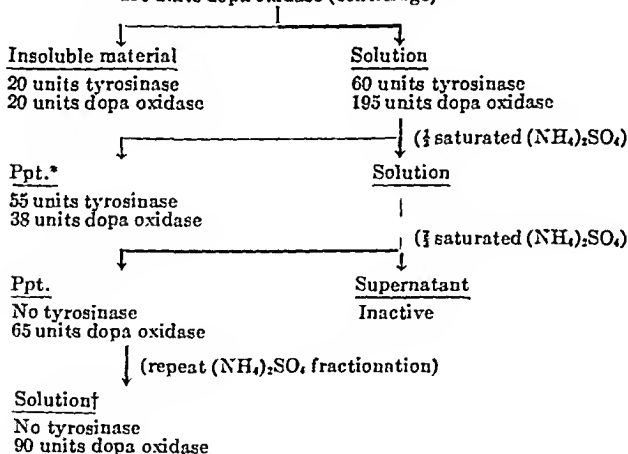
Since the tissue suspension failed to catalyze the oxidation of phenylalanine, it seemed probable that we were not dealing with an amino acid oxidase or amine oxidase, but rather with a phenolic oxidase. The melanoma brei possessed a considerable oxygen absorption in the absence of added substrate; consequently, fractionation and partial purification were desirable before further study of the properties of the enzyme was undertaken. The degree of enzyme activity during the fractionation procedure was followed by the establishment of a convenient activity unit, which was defined as that amount of enzyme required to catalyze the absorption of 1 c.mm. of oxygen per minute by 1 mg. of appropriate substrate.

Fractionation—Trial experiments indicated that, upon centrifugation of the melanoma brei at 20,000 R.P.M. for 10 minutes, most of the enzyme activity remained in solution, the insoluble tissue debris being relatively inactive. Neither dialysis nor precipitation by the addition of ammonium sulfate caused destruction of the enzyme.

On the basis of the trial experiments, the fractionation procedure outlined in the accompanying diagram was adopted. The fraction insoluble

Fractionation of Melanoma Oxidases

70 gm. melanoma ground with sand and Ringer's solution, 65 units tyrosinase,
225 units dopa oxidase (centrifuge)



* Tyrosinase fraction.

† Dopa oxidase fraction.

in one-third saturated ammonium sulfate contained all of the tyrosinase but relatively little of the dopa oxidase activity. This precipitate could not be redissolved and retained a residuum of autoxidation even after dialysis. It will be referred to as the tyrosinase fraction.

The fraction soluble in one-third saturated ammonium sulfate contained 40 per cent of the dopa oxidase activity of the original tissue, but not tyrosinase. It was completely soluble in water and rather deeply pigmented. A 10-fold purification was attained, since this fraction showed 160 units of activity per gm. of dry weight, whereas the original tumor brei contained only 16 units per gm. of dry weight. This enzyme preparation, the dopa oxidase fraction, maintained its activity for more than 2 weeks.

Properties of the Dopa Oxidase Fraction—In Fig. 2 the rate of oxidation of 1 mg. of dihydroxyphenylalanine, catalyzed by varying amounts of the dopa oxidase fraction, is indicated. The maximum reaction velocity is proportional to the amount of enzyme added. When relatively large amounts of the enzyme are present, the rate of oxidation falls off sharply,

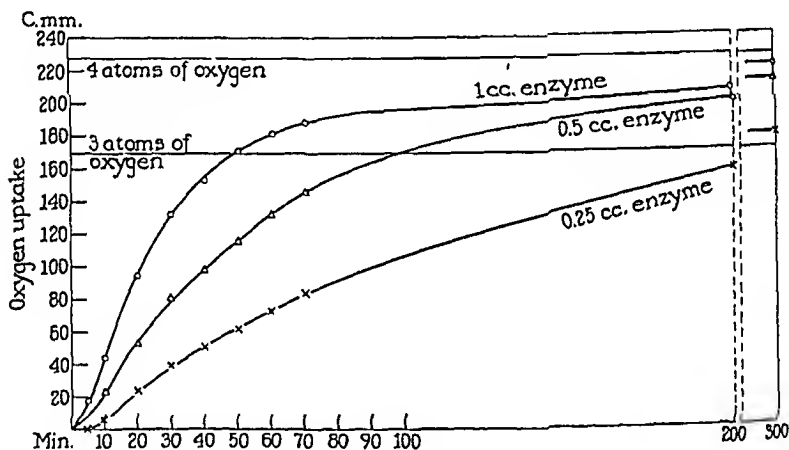


FIG. 2. Action of melanoma oxidase on dopa

indicating exhaustion of substrate, since the rapid uptake of oxygen was resumed when more dopa was added. The total oxygen uptake after completion of the reaction approximates 4 atoms of oxygen per molecule of dopa added as substrate. The end-product of the oxidation of dopa by the melanoma oxidase is the insoluble black pigment, melanin.

The catalytic activity of the dopa oxidase fraction was destroyed by heating for 10 minutes at 100° and was completely inhibited by 0.001 M cyanide ion. The activity was lost at pH 4, much reduced at pH 6, and appeared to be maximum between pH 7 and 8. It should be noted that autoxidation of dopa becomes an important factor in alkaline solution.

The dopa oxidase fraction is apparently very highly specific for dihydroxyphenylalanine. At the concentration of enzyme available, it was completely unable to catalyze the oxidation of tyrosine, hydroquinone,

p-benzylhydroquinone, and *p*-cresol. Catechol was oxidized at one-seventh and adrenalin at one-eighth the rate of dopa.

Properties of the Tyrosinase Fraction—Investigation of the tyrosinase fraction has been less satisfactory, since there is relatively little tyrosinase present in the tumor and since the fractionation procedure apparently rendered the tyrosinase insoluble. This fraction contained the bulk of the originally water-soluble proteins and still absorbed a small amount of oxygen in the absence of substrate. It was possible to demonstrate, however, that the melanoma tyrosinase catalyzed the aerobic oxidation of tyrosine to melanin with the absorption of 5 atoms of oxygen per molecule of substrate.

The catalytic activity of the tyrosinase fraction was destroyed by boiling and completely inhibited by 0.01 *M* but not inhibited by 0.001 *M* KCN.

This tyrosinase at the concentration available failed to oxidize phenol, hydroquinone, and *p*-cresol. The further investigation of its properties must wait until a relatively large quantity of melanoma is available.

Effect of p-Benzylhydroquinone on Melanoma Tyrosinase and Dopa Oxidase—An occupational leucoderma investigated by Schwartz, Oliver, and Warren (13) was found by these authors to be due to the action of monobenzylhydroquinone on the melanoblasts of the skin. Peck and Sobotka (14) found that the action of potato oxidase on tyrosine and dopa is interrupted by this substance at a stage prior to the formation of melanin. When melanoma dopa oxidase is the catalyst, the addition of monobenzylhydroquinone to the dopa solution greatly slows the rate of oxidation, the final product formed being a soluble red pigment rather than the insoluble black melanin otherwise produced. Monobenzylhydroquinone completely inhibits the oxidation of tyrosine by the tyrosinase fraction, no oxygen being absorbed. Mushroom tyrosinase, on the contrary, oxidizes monobenzylhydroquinone itself to a red pigment. Our results in general agree with those of Peck and Sobotka (14) and confirm their explanation of the relationship between monobenzylhydroquinone and leucoderma.

DISCUSSION

In many respects, the melanoma tyrosinase and dopa oxidase show considerable similarity to the tyrosinases isolated from plants and insects. The absorption of 5 and 4 atoms of oxygen per molecule of tyrosine and dopa respectively has been shown by Duliere and Raper (15) to occur when these substances are oxidized to melanin in the presence of meal worm tyrosinase. This finding has been repeatedly confirmed with the use of tyrosinases from such sources as mushrooms and potatoes. The loss of activity with heat and marked reduction of activity in slightly acid solution are properties common to all known tyrosinases. The sensitivity of the

melanoma enzymes to cyanide ion indicates that they are iron- or copper-containing catalysts. All the phenolic oxidases are inactivated by cyanide, and those which have been sufficiently purified have been proved to be copper proteins.

The apparent high specificity of the two fractions is of interest, however, in that it contrasts sharply with the broad specificity of tyrosinases from other sources. The melanoma dopa oxidase, for example, failed to catalyze the oxidation of tyrosine, *p*-benzylhydroquinone, and *p*-cresol and promoted the oxidation of catechol and adrenalin at very slow rates. Mushroom tyrosinase, on the other hand, not only oxidizes all of these compounds, but oxidizes catechol twice as rapidly as it does dopa. The high specificity of the dopa oxidase fraction confirms the findings of Bloch.

It does not necessarily follow from our experiments that these enzymes extracted from a tumor occur in a normal melanoblast. The fact remains, however, that no enzyme system peculiar to tumor has thus far been demonstrated, and until such a discovery is made, we are probably safe in assuming that the production of melanin by this tumor cell is a normal mechanism. The advantages of the transplantable melanoma which is made up essentially of a single cell type, the tumor melanoblast, as a source of enzyme are obvious. It seems conceivable that other neoplasms possessing active and specialized enzymatic function could be utilized in similar studies of other enzyme systems.

SUMMARY

The above experiments demonstrate conclusively that a mouse tumor, which in all probability arose from the skin melanoblast, contains extractable enzymes which catalyze the oxidation of both tyrosine and dihydroxyphenylalanine to melanin. The presence of one of these enzymes, dopa oxidase, has been demonstrated by Bloch to occur in normal mammalian skin, but not in sufficient quantity to allow a detailed study of its properties. The other enzyme, a tyrosinase, has not previously been clearly shown to occur in mammalian tissue.

BIBLIOGRAPHY

1. Bloch, B., *Am. J. Med. Sc.*, **177**, 609 (1929).
2. Laidlaw, G. F., *Am. J. Path.*, **8**, 477 (1932).
3. Durham, F., *Proc. Roy. Soc. London, Series B*, **74**, 310 (1904).
4. Onslow, H., *Proc. Roy. Soc. London, Series B*, **89**, 36 (1915).
5. Yamasaki, Y., *Biochem. Z.*, **147**, 203 (1924).
6. Pugh, C. E. M., *Biochem. J.*, **27**, 475 (1933).
7. Charles, D. R., *Genetics*, **23**, 523 (1938).
8. Russell, W. L., *Genetics*, **24**, 645 (1939).
9. Schlottke, E., *Biol. Zentr.*, **58**, 261 (1938).

10. Neuberg, C., *Biochem. Z.*, **8**, 353 (1908); *Virchows Arch. path. Anat.*, **192**, 514 (1908).
11. Alsberg, C. L., *J. Med. Research*, **16**, 117 (1907).
12. Harding, H. E., and Passey, R. D., *J. Path. and Bact.*, **33**, 417 (1930).
13. Schwartz, L., Oliver, E. A., and Warren, L. H., *Pub. Health Rep., U. S. P. H. S.*, **55**, 1111 (1940).
14. Peck, S. M., and Sobotka, H., *J. Investigative Dermat.*, **4**, 325 (1941).
15. Duliere, W. L., and Raper, H. S., *Biochem. J.*, **24**, 239 (1930).

THE EFFECT OF ANAEROBIC CONDITIONS AND RESPIRATORY INHIBITORS ON THE IN VITRO PHOSPHOLIPID FORMATION IN LIVER AND KIDNEY WITH RADIOACTIVE PHOSPHORUS AS INDICATOR

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In a previous communication from this laboratory the formation of phospholipid by surviving liver slices was demonstrated with the aid of radioactive phosphorus (1). Thin slices of rat liver were suspended in a bicarbonate-Ringer's solution medium containing labeled inorganic phosphorus; at the end of 1 or 2 hours it was found that the phospholipid isolated from the mixture contained appreciable amounts of radioactive phosphorus. Evidence was presented to show that this P^{32} was lipid phosphorus and not inorganic phosphorus. Although new phospholipid was being formed during the course of the experiment, the net effect was a decrease in the total quantity of phospholipid present. No formation of phospholipid was detected in liver preparations in which tissue organization had been disrupted by mincing or homogenizing.

The linkages in the phospholipid molecule are such that the hydrolysis of this molecule would be expected to occur with a decrease in free energy. The hydrolysis of the phosphate-alcohol bond in glycerol phosphate has been shown to have a ΔF° value of -2280 calories (2, 3), and the value for the hydrolysis of a similar bond in the phospholipid molecule would not be expected to differ greatly. There are two alcohol-phosphate bonds (both involving the same P atom) in the phospholipid molecule; hence the ΔF° for the hydrolysis of the phospholipid molecule to inorganic phosphate would be expected to be at least about -5000 calories. In applying these considerations to the conditions in the tissue slice, it should be noted that the system is heterogeneous and one in which the concentrations of the various components involved in phospholipid formation are unknown. However, even under the conditions of the tissue slice, it is very likely that the hydrolysis of phospholipid to inorganic phosphate proceeds with an appreciable decrease in free energy and that the tendency of the reaction is far in the direction of hydrolysis. It is not surprising, therefore, that in the experiments carried out here there is a decrease in total phospholipid during the course of a run. Moreover, it would be expected that the formation of phospholipid from inorganic phosphate would be an energy-consuming reaction. Hence, in the synthesis of phospholipid from inorganic phos-

phate, coupling with energy-producing reactions should be suspected. The experimental evidence cited below suggests that such coupling actually exists. Anaerobic conditions and the respiratory inhibitors, cyanide, sulfide, azide, and carbon monoxide, were all found to inhibit the incorporation of radioactive inorganic phosphate into phospholipid.

Effect of Anaerobic Conditions—The effects of anaerobiosis on phospholipid synthesis are recorded in Table I. In both liver and kidney slices it is evident that phospholipid formation is greatly impaired in the absence of oxygen. In most cases the formation was inhibited to the extent of 90 per cent. The slight formation of phospholipid that did occur in the absence of oxygen was not negligible and was well outside the range of experimental error. However, this small residual synthesis of phospholipid

TABLE I
Effect of Anaerobic Conditions on Phospholipid Formation

Rat No.	Tissue	Period of incubation	Per cent of added P ³² recovered as phospholipid per gm. tissue				Per cent inhibition
			Control, oxygen present		Anaerobic conditions		
			Wet weight	Dry weight	Wet weight	Dry weight	
1	Liver	<i>hrs.</i> 4	9.2	40	0.60	2.6	93
2	"	4	6.5	29	0.53	2.3	92
3	"	2	3.2	14	0.44	1.9	86
4	"	2	3.8	17	0.21	0.92	94
5	Kidney	4	4.7	26	0.058	0.32	99
6	"	4	3.4	19	0.41	2.3	88
7	"	2	3.3	18	0.17	0.95	95
8	"	2	3.5	20	0.80	4.5	77

should not be ascribed to a non-oxygen-utilizing system, because the presence of minute amounts of oxygen in the tissue slice or in the gas mixture was not ruled out.

Effect of Cyanide—The influence of various concentrations of NaCN on phospholipid formation is recorded in Table II. Phospholipid formation in surviving liver and kidney slices is extremely sensitive to the presence of NaCN. Concentrations as low as 0.001 M NaCN inhibited the formation in liver slices to the extent of about 90 per cent.

It has been pointed out above that, although phospholipid synthesis can be detected in liver slices suspended in the bicarbonate-Ringer's solution, the net effect during the course of a run is a decrease in the total phospholipid. A loss in phospholipid can be shown in two ways: (1) by the determination of the total phospholipid per gm. of slice before and at the

end of a run; (2) by the use of slices from a rat that had received an injection of radioactive Na_2HPO_4 several hours prior to excision of the liver; these slices contained preformed radioactive phospholipid and the difference in the amounts of radiophospholipid present before and at the end of a run is also a measure of phospholipid breakdown. Both methods have been used to measure the decomposition of phospholipid in slices prepared from a single liver (1).

The effects of cyanide on both types of decomposition were investigated in the present study. The results are recorded in Table III. Cyanide did

TABLE II
Effect of Cyanide on Phospholipid Formation (Period of Incubation 2 Hours)

Rat No.	Tissue	Concentration of cyanide	Per cent of added P ³² recovered as phospholipid per gm. tissue				Per cent inhibition
			Control, cyanide absent		Cyanide present		
			Wet weight	Dry weight	Wet weight	Dry weight	
1	Liver	0.03	1.6	7.0	0.065	0.29	96
2	"	0.03	2.4	11	0.011	0.048	100
3	"	0.01	1.7	7.5	0.098	0.43	94
4	"	0.01	2.2	9.7	0.015	0.066	99
5	"	0.01	2.2	9.7	0.057	0.25	97
6	"	0.01	2.2	9.7	0.29	1.3	87
7	"	0.01	3.0	13	0.14	0.62	95
8	"	0.005			0.054	0.24	
9	"	0.003	2.1	9.2	0.24	1.1	89
10	"	0.003	2.9	13	0.25	1.1	92
11	"	0.001	3.2	14	0.32	1.4	90
11	"	0.001	3.2	14	0.39	1.7	88
12	Kidney	0.01	2.3	13	0.00	0.00	100
13	"	0.01	1.9	11	0.00	0.00	100
14	"	0.005	1.8	10	0.00	0.00	100
15	"	0.005	1.5	8.4	0.00	0.00	100

interfere with the breakdown of phospholipid in liver slices that were suspended for 3 hours in the bicarbonate-Ringer's solution. It is thus evident that in doses that inhibited lipid phosphorylation in liver slices cyanide had no effect on phospholipid decomposition.

Effect of Sulfide—The effect of different concentrations of H_2S on phospholipid formation is shown in Table IV. It is evident that H_2S is practically as powerful as cyanide in inhibiting lipid phosphorylation in liver and kidney slices. The degree of inhibition averaged 90 per cent in the case of liver slices; kidney slices appeared to be more sensitive than liver.

Effect of Azide—The effect of 0.005 M sodium azide (NaN_3) on phospholipid formation is recorded in Table V. The inhibitory effect is definite, but considerable variation was observed in the degree of inhibition. This varied from 35 to 95 per cent in liver and from 53 to 95 per cent in kidney.

TABLE III
Effect of Cyanide on Decomposition of Phospholipid in Liver Slices

Experiment No.	Method of measuring phospholipid decomposition	Per cent decomposition in 3 hrs	
		Cyanide absent	0.01 M cyanide
1*	Radiophospholipid†	14	25
	Total phospholipid‡	22	26
2*	Radiophospholipid	14	19
	Total phospholipid	19*	21
3§	Radiophospholipid	21	28
	Total phospholipid	20	28
4§	Radiophospholipid	24	24
	Total phospholipid	21	29

* Slices prepared from the same liver.

† As measured by loss of initially present radiophospholipid.

‡ As measured by loss of initially present total phospholipid.

§ Slices prepared from the same liver.

TABLE IV
Effect of Sulfide on Phospholipid Formation (Period of Incubation 2 Hours)

Rat No.	Tissue	Saturated H ₂ S solution added to bath	Per cent of added P ³² recovered as phospholipid per gm. tissue				Per cent inhibition
			Control, H ₂ S absent		H ₂ S present		
			Wet weight	Dry weight	Wet weight	Dry weight	
1	Liver	cc.	2.5	11	0.23	1.0	91
2	"	0.5	2.1	9.2	0.24	1.1	89
3	"	0.10	2.0	8.8	0.21	0.92	90
4	Kidney	0.50	2.0	11	0.00	0.00	100
5	"	0.25	2.0	11	0.00	0.00	100
6	"	0.10	2.3	13	0.04	0.22	98

The inhibitory effect of azide under the present conditions was not as pronounced as that of cyanide or H_2S .

Effect of Carbon Monoxide—The classical experiments of Warburg (4) on the influence of carbon monoxide on respiration suggested its use here. The first experiment was carried out with a gas mixture containing 80 per cent CO , 19 per cent O_2 , and 1 per cent CO_2 ; i.e., a carbon monoxide-oxygen

TABLE V

Effect of 0.005 M Azide on Phospholipid Formation (Period of Incubation 2 Hours)

Rat No.	Tissue	Per cent of added P ³² recovered as phospholipid per gm. tissue				Per cent inhibition
		Control, azide absent		Azide present		
		Wet weight	Dry weight	Wet weight	Dry weight	
1	Liver	4.8	21	0.99	4.4	79
2	"	5.8	26	0.31	1.4	95
3	"	2.5	11	0.87	3.8	65
4	"	1.7	7.5	1.1	4.8	35
5	"	4.8	21	0.61	2.7	87
6	"	4.8	21	0.73	3.2	85
7	"	2.9	13	0.38	1.7	87
8	Kidney	3.5	20	0.28	1.6	92
9	"	3.9	22	0.38	2.1	90
10	"	1.9	11	0.90	5.0	53
11	"	1.7	9.5	0.71	4.0	58
12	"	5.0	28	0.27	1.5	95

TABLE VI

Effect of Carbon Monoxide on Phospholipid Formation (Period of Incubation 2 Hours)

Rat No.	Tissue	Per cent composition of gas mixture above radioactive Ringer's bath*		Per cent of added P ³² recovered as phospholipid per gm. tissue				Per cent inhibition
		Control, CO absent	CO present	Control, CO absent		CO present		
				Wet weight	Dry weight	Wet weight	Dry weight	
1	Liver	Air	80% CO, 19% O ₂ , 1% CO ₂	2.7	12	3.1	14	None
2	"	"	" "	4.2	18	4.2	18	"
3	"	"	" "	2.7	12	2.7	12	"
4	"	90% N ₂ , 5% O ₂ , 5% CO ₂	90% CO, 5% O ₂ , 5% CO ₂	2.4	11	1.5	6.6	38
5	"	" "	" "	1.4	6.2	0.86	3.8	39
6	"	" "	" "	2.5	11	1.6	7.0	36
7	"	" "	" "	2.4	11	1.9	8.4	21
8	"	" "	" "	2.2	9.7	1.5	6.6	32
9	Kidney	Air	80% CO, 19% O ₂ , 1% CO ₂	2.1	12	1.6	9.0	24
10	"	"	" "	1.5	8.4	1.1	6.2	27
11	"	90% N ₂ , 5% O ₂ , 5% CO ₂	90% CO, 5% O ₂ , 5% CO ₂	1.2	6.7	0.27	1.5	78
12	"	" "	" "	1.6	9.0	0.30	1.7	81
13	"	" "	" "	0.97	5.4	0.43	2.4	56
14	"	" "	" "	1.2	6.7	0.17	0.95	86
15	"	" "	" "	1.3	7.3	0.17	0.95	87
16	"	" "	" "	0.90	5.0	0.12	0.67	87

* Uncorrected for water vapor.

ratio of 4. In control experiments air was used as the gas mixture above the tissue slices. Table VI shows that phospholipid formation in *liver* slices is not inhibited by CO when the CO:O₂ ratio is as low as 4. In surviving kidney slices, however, the presence of such a gas mixture did have some inhibitory effect.

Before we proceeded to experiments in which the value of the carbon monoxide-oxygen ratio was increased, it was necessary to determine how much the oxygen tension above the slices could be lowered without re-

TABLE VII

Effect of Various Oxygen Tensions on Phospholipid Formation in Liver Slices (Period of Incubation 2 Hours)

Gas mixture in atmosphere above slices*			Per cent of added P ³² recovered as phospholipid per gm., wet weight		
Per cent O ₂	Per cent N ₂	Per cent CO ₂	Rat 1	Rat 2	Rat 3
0	95	5	0.18	0.15	0.79
5	90	5	1.6	1.7	2.7
10	85	5	2.5	2.3	2.9
21†	79		3.3	2.9	4.0

* Uncorrected for water vapor.

† Air used as atmosphere above slices.

TABLE VIII

Effect of Light on CO Inhibition of Phospholipid Formation in Liver Slices (Period of Incubation 2 Hours)

Rat No.	Per cent of added P ³² recovered as phospholipid per gm. tissue						Per cent inhibition	
	Control		CO in light		CO in dark		CO in light	CO in dark
	Wet weight	Dry weight	Wet weight	Dry weight	Wet weight	Dry weight		
1	1.9	8.4	1.4	6.2	1.1	4.8	26	42
2	1.5	6.6	0.87	3.8	0.59	2.6	42	61
3	2.1	9.2	1.1	4.8	0.68	3.0	48	68

ducing phospholipid formation too greatly. Slices from the same liver were subjected to four different O₂ tensions; the results obtained on the livers of three different animals are recorded in Table VII. The results show that considerable formation of phospholipid still occurred when the atmosphere above the solution contained only 5 per cent oxygen.

When the carbon monoxide-oxygen ratio was raised to a value of about 18, its inhibitory effect on phospholipid formation in both liver and kidney slices was definite (Table VI). In these experiments the gas mixture consisted of 90 per cent CO, 5 per cent O₂, and 5 per cent CO₂, whereas the con-

trol gas mixture consisted of 90 per cent N_2 , 5 per cent O_2 , and 5 per cent CO_2 . The results show also that lipid phosphorylation in *kidney* slices is more sensitive to the presence of CO than that in liver slices. The degree of inhibition in liver slices averaged about 35 per cent, whereas in kidney slices the average inhibition was about 80 per cent.

In view of the influence of light on CO inhibition of respiration (4) it became of interest to determine whether light could modify the inhibitory effects of CO on phospholipid formation. The results recorded in Table VIII show that the inhibitory effect of CO is more pronounced in the dark than in the presence of strong light.

DISCUSSION

Cellular Oxidations and Phospholipid Formation in Vitro—The experimental evidence cited above suggests that the aerobic oxidations of the cell provide the immediate energy required for the formation of phospholipids from inorganic phosphate. The respiratory inhibitors, cyanide, azide, H_2S , and CO, inhibit the activity of cytochrome oxidase (5); the results suggest, therefore, that the cytochrome-cytochrome oxidase system is the aerobic oxidative system involved here. The possible existence of an appreciable source of anaerobic energy that could be used for phospholipid formation is, however, not ruled out.

Although inorganic phosphorus is readily incorporated into phospholipid, the reactions involved in this conversion are not known. It is possible, however, to rule out certain mechanisms that will be designated here "exchange reactions." "Exchange" is defined here as the simple interchange of atoms or radicals between two different molecules such as the interchange of the phosphate radical between inorganic phosphate and the phospholipid molecule. To be classified as an exchange reaction in this sense, interchange must take place without the addition of energy from an external source or from energy-producing reactions.

Two types of exchange mechanisms that conceivably may be involved in the formation of radioactive phospholipid will be considered. (a) *Exchange through collision*. Collision between an inorganic phosphate molecule and a phospholipid molecule could result in an interchange of P atoms or of phosphate radicals. Such a collision could result in the incorporation of radioactive inorganic phosphate into a phospholipid molecule. The observations recorded above, however, show that the formation of radioactive phospholipid does not take place in this manner. The failure of homogenized liver to form radioactive phospholipid, as well as the inhibitory effects of anaerobic conditions and respiratory inhibitors, offers convincing evidence that exchange through collision does not occur to any appreciable extent. (b) *Exchange due to reversibility of reactions*. Since a chemical reac-

tion may be considered to be the net result of two opposing reactions proceeding simultaneously in opposite directions at different rates, it is conceivable that radioactive inorganic phosphorus becomes converted to phospholipid through the slight reversibility of the decomposition reaction. Although the rate of decomposition of phospholipid under these conditions is quite slow (10 to 20 per cent in 3 hours (1)) and the equilibrium for this reaction is probably far in the direction of hydrolysis, a slight reversibility of this kind must be kept in mind, particularly in view of the sensitivity of the radioactive labeling procedure. This mechanism of exchange, however, can also be ruled out, in view of the results obtained with respiratory inhibitors. The fact that the formation of radioactive phospholipid in the liver is almost completely inhibited by the presence of cyanide, whereas the decomposition is unimpaired under these conditions, is strong evidence that no such exchange occurs under the present conditions (Table III).

EXPERIMENTAL

The extraction procedure for phospholipid has been reported (6); in this procedure contaminating inorganic radioactive phosphorus is diluted at every possible step with inert inorganic phosphorus. The only difference between the method used for brain tissue (6) and the one used here for liver and kidney was in the number of washings with saturated non-radioactive Na_2HPO_4 employed for the removal of the last traces of contaminating radioactive inorganic phosphate. Much less difficulty is encountered in removing such contamination from liver and kidney extracts than from brain extracts; only one washing was found necessary in the experiments reported here. The procedures for the precipitation of phospholipid and the measurement of their radioactivity have been described elsewhere (7).

The procedure employed here for the isolation of phospholipids has been tested many times and shown to be satisfactory for the separation of radio-phospholipid from radioactive inorganic phosphorus. The phospholipid isolated in a zero time experiment (1, 6) contained less than 10 radioactive counts. This number of counts is negligible when compared with (a) the number of counts, namely 10^5 , added to the bath as inorganic radiophosphorus, and (b) the number of counts, namely 700 or more, found as phospholipid P^{32} at 2 and 4 hours. This observation clearly demonstrates that it is possible to distinguish phospholipid phosphorus from inorganic phosphorus by the method.

Anaerobic Experiments—Anaerobic experiments (Table I) were carried out in the presence of a gas mixture containing 95 per cent nitrogen and 5 per cent CO_2 , the control experiments in the presence of a mixture of 95 per cent oxygen and 5 per cent CO_2 . Slices from a single liver or from both kidneys of a single rat were collected in a Petri dish containing a non-radio-

active bicarbonate-Ringer's solution that previously had been saturated with the nitrogen- CO_2 gas mixture. Slices from the same liver or from the kidneys of a single rat were used in both nitrogenated and oxygenated media. In this way phospholipid synthesis was compared under aerobic and anaerobic conditions in slices obtained from the same organ. The use of slices from a single liver or from the same pair of kidneys for both experimental and control observations was adhered to throughout this investigation.¹

Approximately 300 mg. of slices were then removed from the Petri dish, blotted on a filter paper, weighed on a small piece of cellophane, and then transferred to a flask containing 5 cc. of either an oxygenated or a nitrogenated bicarbonate-Ringer's solution. The same bicarbonate-Ringer's solution containing radioactive phosphorus (1) was used in both cases; in the former it was saturated with a gas mixture containing 95 per cent oxygen and 5 per cent CO_2 , in the latter with a mixture of 95 per cent nitrogen and 5 per cent CO_2 .² Both solutions were adjusted to pH 7.4 before the addition of the slices. To insure anaerobic conditions, the reaction flasks were flushed out with the appropriate gas mixture both before and after the addition of the slices. In the case of the controls, the atmosphere above the bicarbonate-Ringer's solution was flushed out with a 95 per cent O_2 -5 per cent CO_2 mixture after the addition of the slices.

Preparation of Radioactive Bicarbonate-Ringer's Solution Containing Cyanide—Stock solutions of NaCN varying from 0.1 to 0.5 M were first prepared. When required, an aliquot of a stock cyanide solution was neutralized with an amount of concentrated HCl calculated to bring its pH to approximately 7.4. Final adjustments to pH 7.4 were made with dilute acid or base. The various concentrations of cyanide recorded in Table II were then prepared by adding suitable amounts of this neutralized NaCN solution to enough oxygenated radioactive bicarbonate-Ringer's solution, pH 7.4, to make a final volume of 5 cc. in all cases. The amount of neutralized NaCN usually added was 0.1 to 0.25 cc., in a few cases 0.5 cc. The effect of these small volumes on the tonicity of the medium was considered negligible.

¹ The percentage recovery of radiophospholipid varied a good deal from animal to animal. Duplicate runs with the use of slices prepared from a single liver or from the two kidneys obtained from a single rat, however, checked closely. Hence, in order to determine the effect of an agent upon the recovery of radiophospholipid, it was desirable to use slices prepared from the same liver or same pair of kidneys in both the control bath and in the bath containing the agent under investigation. By the use of slices prepared from a single liver or a single pair of kidneys possible differences in the specific activity of the inorganic phosphorus inside the slices are minimized.

² A commercial preparation containing not more than 0.3 per cent oxygen.

Preparation of Radioactive Bicarbonate-Ringer's Solution Containing Sulfide—A saturated solution of H_2S was prepared by bubbling the gas through distilled water at 0° for an hour. The pH of this solution was then adjusted to neutrality by the addition of 1.5 N NaOH. From this neutral solution aliquots of 0.1, 0.25, and 0.5 cc. were removed and added to enough oxygenated radioactive Ringer's solution of pH 7.4 to make the final volume 5 cc. (Table IV).

Preparation of Radioactive Bicarbonate-Ringer's Solution Containing Azide—The experiments with sodium azide, NaN_3 , (Table V) were carried out similarly to those performed with cyanide. 0.25 M stock solution of NaN_3 was first prepared and the pH adjusted to approximately 7.4. 0.2 cc. portions of this neutral solution were added to 4.8 cc. of oxygenated radioactive Ringer's solution of pH 7.4. This provided a concentration of azide in the Ringer's bath of 0.005 M. Slices from the same liver or pair of kidneys were used for both azide and control experiments.

CO Experiments—Carbon monoxide was prepared by the action of formic acid on hot concentrated sulfuric acid. The gas train contained a strong KOH solution through which the gas was bubbled to remove SO_2 that might have been formed. The CO was collected in a graduated 2 liter aspirator bottle and the required amounts of oxygen and carbon dioxide added from tanks containing these gases. In this manner various mixtures of CO, O_2 , and CO_2 were prepared.

The gas mixture contained in the aspirator bottle was introduced into the reaction flasks by means of an arrangement similar to that described by Keilin (8). Each flask containing 5 cc. of oxygenated Ringer's solution was filled with the proper gas mixture and shaken in the constant temperature bath for about 15 minutes before the addition of the slices. In this way equilibrium was established between the gas mixture and the solution. After the addition of the slices, the vessel was again flushed and filled with the proper gas mixture and then placed in the constant temperature bath for 2 hours.

To test the effect of light on CO inhibition, the experiment was carried out as described above. Livers from three different animals were used, and from each liver enough slices were prepared for three baths, a control experiment, a CO dark experiment, and a CO light experiment. Dark conditions were obtained by covering the reaction flasks thoroughly with black paint. All reaction flasks were placed in the same thermostat and exposed to strong light from a reflector lamp containing a 150 watt bulb. The control flasks were not darkened; a previous experiment had shown that the control reaction was not light-sensitive. Two gas mixtures were used: 90 per cent CO, 5 per cent O_2 , and 5 per cent CO_2 , to test the effects of light on CO inhibition; 90 per cent N_2 , 5 per cent O_2 , and 5 per cent CO_2 , for the control.

Experiments on Decomposition of Phospholipids—The breakdown of radiophospholipid and total phospholipid in liver slices kept in a bicarbonate-Ringer's solution medium was determined as follows: Rats were injected with radioactive Na_2HPO_4 solution and their livers removed about 6 hours after the injection. The livers were sliced in the usual manner and enough slices for six separate baths prepared from each liver. Two samples were weighed and extracted immediately for phospholipids; total phospholipid and radiophospholipid were determined. These measurements provided initial values. Two other samples were placed in 5 cc. of a non-radioactive bicarbonate-Ringer's solution, the chemical composition of which was identical with that employed in other experiments. Two more samples were placed in 5 cc. of a similar non-radioactive Ringer's solution containing 0.01 M NaCN. These four samples were placed in the constant temperature bath for 3 hours, the conditions being exactly the same as those for phospholipid formation. At the end of 3 hours all four samples were extracted in the usual manner and their content of both total and radiophospholipid determined (1).

The radioactive phosphorus used in this investigation was prepared by members of the Radiation Laboratory under the direction of Professor E. O. Lawrence, to whom our thanks are due.

SUMMARY

1. Anaerobic conditions and the respiratory inhibitors, cyanide, azide, H_2S , and CO, inhibit the formation of phospholipid by liver and kidney slices as measured with radioactive phosphorus. Lipid phosphorylation is inhibited to the extent of about 90 per cent by cyanide, H_2S , and anaerobiosis. The inhibitory effect of CO is more pronounced in the dark than in the presence of strong light. These observations suggest that the cytochrome-cytochrome oxidase system is involved in phospholipid formation *in vitro*.

2. The mechanism of lipid phosphorylation is discussed. Coupling with an energy-producing mechanism in the cell is suggested.

BIBLIOGRAPHY

1. Fishler, M. C., Taurog, A., Perlman, I., and Chaikoff, I. L., *J. Biol. Chem.*, **141**, 809 (1941).
2. Kay, H. D., *Biochem. J.*, **22**, 855 (1928).
3. Lipmann, F., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **1**, 99 (1941).
4. Warburg, O., *Z. angew. Chem.*, **45**, 1 (1932).
5. Green, D. E., *Mechanisms of biological oxidations*, Cambridge, **32** (1940).
6. Fries, B. A., Schachner, H., and Chaikoff, I. L., *J. Biol. Chem.*, **144**, 59 (1942).
7. Perlman, I., Ruben, S., and Chaikoff, I. L., *J. Biol. Chem.*, **122**, 169 (1937-38).
8. Keilin, D., *Proc. Roy. Soc. London, Series B*, **104**, 206 (1929).

CATECHOLASE (TYROSINASE):* AN IMPROVED METHOD OF PREPARATION

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A special interest in the enzyme tyrosinase has been evoked by the claim of Schroeder and Adams (1) that this phenoloxidase will, on subcutaneous injection, lower the blood pressure of experimental animals with hypertension. The methods at present available for the preparation of this enzyme are rather involved and time-consuming (2, 3). It seemed to us, therefore, desirable to work out a more convenient procedure by which one could obtain in good yield tyrosinase preparations of fairly high purity. In the following we wish to report briefly on our method of preparation.

EXPERIMENTAL

Determination of Enzymic Activity—Mushroom extracts have been found to exert the following two enzymic actions, (a) oxidation of monophenols and (b) oxidation of *o*-dihydric phenols such as catechol. The latter effect has been called the catecholase activity. Whether these two effects are produced by one or two different enzymes has not yet been definitely established. We have followed the enzymic activity of the various fractions according to the method of Adams and Nelson (4), using catechol as a substrate, 1 unit of catecholase activity being defined as the amount of enzyme required to cause the rate of oxygen uptake to equal 10 c.mm. per minute.

Preparation of Enzyme—10 pounds of the common mushroom, *Psalliota campestris*, were finely ground into 7.5 liters of acetone which had been previously chilled with dry ice. The mixture was stirred for 5 minutes, filtered by suction, and the filtrate discarded. The mushroom residue was pressed out in a hydraulic press, then mixed with dry ice, and allowed to stand for 5 hours. After most of the remaining dry ice was removed, 6 liters of 0.2 saturated ammonium sulfate (140 gm. of ammonium sulfate per liter of water) were added gradually. The mixture was stirred for 1 hour, allowed to stand overnight at room temperature, stirred for another hour the following morning, and then filtered through cheese-cloth. The residue was pressed out in a hydraulic press and discarded.

The 0.2 saturated ammonium sulfate extract was centrifuged, the super-

* The term catecholase is preferable to tyrosinase, but the latter, however, is still in general use.

natant brought to 0.5 saturated ammonium sulfate,¹ a little celite added, and the mixture allowed to stand overnight at room temperature. The precipitate was filtered by suction through a celite mat, washed with 0.5 saturated ammonium sulfate solution, and extracted with about 400 ml. of $M/15$ phosphate buffer (pH 7.3) in 300 ml. and 100 ml. portions. After centrifugation, the extract was brought to pH 4.9 to 5.0 with glacial acetic acid and allowed to stand overnight in the refrigerator. The precipitate which formed was removed by centrifugation, and the supernatant dialyzed in the refrigerator for 3 days. (In this and the subsequent dialyses double distilled water was used and was changed twice daily.) The precipitate, which appeared on dialysis, was either removed by centrifugation or by filtration through a No. 2 Whatman filter paper. (This grade of paper was used in all subsequent filtrations.)

The filtrate was brought to 0.3 saturated ammonium sulfate and allowed to stand overnight at room temperature. The precipitate was removed by filtration, and the solution brought to 0.4 saturated ammonium sulfate. After standing overnight, the precipitate was filtered and extracted with about 50 ml. of 0.2 M acetate buffer (pH 5.0), and the extract dialyzed for 2 days. Any precipitate which formed during dialysis was removed by centrifugation, and the supernatant brought to 0.3 saturated ammonium sulfate and allowed to stand overnight. After filtration, the solution was brought to 0.35 saturated ammonium sulfate and allowed to stand overnight. The 0.3 to 0.35 saturated ammonium sulfate precipitate was separated by filtration and dissolved in about 50 ml. of 0.2 M acetate buffer (pH 5.0), and the solution dialyzed for 3 days. Any precipitate formed during dialysis was removed by centrifugation.

The solution thus obtained is slightly yellow in color and was usually found to contain about 500 catecholase units per mg. of dry weight of organic matter and 800 units per ml. The yield is 5000 to 6000 units per pound of mushrooms and represents about one-third of the total potency present in the original 0.2 saturated ammonium sulfate extract. It was observed that the amount of enzyme obtained in this extract varied with different grades of mushrooms. The activity of this first extract was about 15 units per mg. of dry weight of organic matter. A 35-fold purification has thus been achieved.

SUMMARY

An improved procedure for the preparation of catecholase (tyrosinase) from mushrooms is described. The preparations thus obtained were found to contain about 500 catecholase units per mg. of dry organic weight.

¹ The proper concentration of ammonium sulfate was acquired by the gradual addition of the calculated amount of the salt to the solution with stirring on the basis that 70 gm. of ammonium sulfate in 100 ml. of water give a saturated solution.

BIBLIOGRAPHY

1. Schroeder, H. A., and Adams, M. H., *J. Exp. Med.*, **73**, 531 (1941).
2. Keilin, D., and Mann, T., *Proc. Roy. Soc. London, Series B*, **125**, 187 (1938).
3. Ludwig, B. J., and Nelson, J. M., *J. Am. Chem. Soc.*, **61**, 2601 (1939).
4. Adams, M. H., and Nelson, J. M., *J. Am. Chem. Soc.*, **60**, 2474 (1938).

STUDIES ON THE SUCCINOXIDASE SYSTEM OF RAT LIVER IN RIBOFLAVIN DEFICIENCY*

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It has been previously reported that one or more components of the succinoxidase system of rat liver are affected by the dietary intake of riboflavin (1). This observation supported the suggestion (2) that succinic dehydrogenase may be a flavoprotein analogous to the yeast cytochrome *c* reductase of Haas, Horecker, and Hogness (3).

The marked stimulatory effect of calcium ions upon the succinoxidase activity of fresh rat tissues has been demonstrated by Axelrod *et al.* (4) and in the studies on the succinoxidase content in riboflavin deficiency calcium ions were always added to the *in vitro* system in order to attain the maximal enzymatic activity. Under the conditions employed in these experiments the addition of aluminum ions in the absence of added calcium had no stimulatory effect. These observations were confirmed by Potter and Schneider (5), who were able to show, however, that aluminum could overcome a dilution effect observed in the succinoxidase system in the presence of added cytochrome *c* and calcium ions. The significance of aluminum in the succinoxidase system had been noted previously by Horecker *et al.* (6). These observations upon the aluminum effect made it necessary to reinvestigate the status of our assay procedure which had been carried out in the absence of added aluminum ions.

Cytochrome oxidase, being an essential component of the succinoxidase system, could conceivably be a limiting factor in the aerobic oxidation of succinate. In order to check this possibility, the cytochrome oxidase content of the tissue was studied simultaneously with the determination of the total succinoxidase activity. The elimination of cytochrome oxidase as a limiting factor would tend to emphasize the validity of the assay procedure as a measure of the succinic dehydrogenase content of tissue. In conjunction with these experiments it became of interest to study the relationships of other components of the succinoxidase system, *i.e.* calcium and cytochrome *c*, in a riboflavin deficiency.

It had been observed that the increase in succinoxidase activity of the

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liver following riboflavin therapy was always greatest in those rats whose food intake was restricted to that of the animals on the riboflavin-low basal diet. Such controlled feeding experiments were originally instituted in order to avoid, if possible, complications due to increased growth following riboflavin therapy. However, animals subsisting on a restricted food intake are actually in a chronically starved state and the possibility exists that changes in various constituents of the liver correlated with changes in the total size of the liver may drastically affect enzymatic measurements, *i.e.* $Q_{0.0}$, computed solely on a unit of dry weight basis. With this in mind, glycogen, fat, and protein analyses were made on all livers in which the succinoxidase activity was studied. In addition, the total dry weight of each liver was determined and the succinoxidase activity of the total organ was calculated in arbitrary terms. It was thought that data of this type might be of considerable help in arriving at the most valid interpretation of the experimental results.

Finally, in order to study the specificity of the riboflavin effect upon the succinoxidase system, the livers of biotin- and pantothenic acid-deficient rats were assayed for their succinoxidase contents. These animals were receiving ample amounts of riboflavin and their pathological state could be corrected by the addition of either biotin or pantothenic acid, respectively.

EXPERIMENTAL

Treatment of Animals—Weanling male, albino rats were placed on the riboflavin-low ration which has been fully described in a previous publication (7).¹ In Series II the procedure was varied somewhat in that the daily vitamin supplements² were given orally in supplement dishes. In this manner, the rats were assured of an excessive vitamin intake during periods in which the food intake was below normal. The growth on this basal ration is very poor and ceases entirely after 2 weeks. Thereafter a weight plateau is maintained for a period of at least 12 weeks. Supplementation of this basal ration with riboflavin results in normal growth. The animals were weighed weekly and were fed *ad libitum* unless otherwise designated.

Series I—The animals in this series were divided into four groups. Group 1 received only the riboflavin-low ration for 10 weeks. From the beginning of the experiment Group 2, consisting of twelve rats, received

¹ The diet is referred to as Ration B in this publication.

² 30 γ of thiamine and pyridoxine, 150 γ of pantothenic acid, 15 mg. of choline, 500 γ of nicotinic acid, and liver filtrate equivalent to 0.4 gm. of the original liver extract. We are indebted to Merck and Company, Inc., for generous supplies of the synthetic vitamins.

the basal diet supplemented with 300 γ of riboflavin per 100 gm. of ration. In an attempt to determine whether excessive dosages of riboflavin would affect the succinoxidase content of liver, six rats from this group were given, in addition, 500 γ of riboflavin daily for 2 weeks prior to sacrifice. The average weekly gain in weight over the 9 week experimental period was 28 gm. for the twelve rats in this group. Groups 3 and 4 were maintained on the basal ration for 9 weeks before riboflavin therapy was instituted. Both groups then received 100 γ of riboflavin daily *per os* over a 2 week period. The rats in Group 3 were fed *ad libitum* during the period of therapy and showed an average weekly gain of 34 gm., while those in Group 4, whose daily food intake was restricted to that of the basal group (4 gm. of ration), gained an average of 4 gm. per week.

Series II—The animals in this series were divided into six groups. Group 1 received only the riboflavin-low ration for 12 to 14 weeks. Groups 2, 3, and 4 were maintained on the basal ration for 7, 10, and 14 weeks respectively before the initiation of riboflavin therapy (100 γ of riboflavin daily *per os*). Group 2 was fed *ad libitum* during a 6 week period of therapy and showed the usual weight response. The daily food intake of the rats in Groups 3 and 4 was restricted to 3 gm. during the period of therapy, which was 4 weeks for Group 3 and 3 days for Group 4. No weight gains were observed in Groups 3 and 4.

In order to study the effect of chronic starvation upon the succinoxidase activity of rat liver, twelve weanling male, albino rats were fed the riboflavin-low ration *ad libitum* and the daily vitamin supplements were furnished in supplement dishes. In addition all of the rats were given 100 γ of riboflavin daily during the entire course of this experiment. After 1 week on this regimen six of the rats (Group 5) were sacrificed and the food intake of the remaining rats was limited to amounts which did not permit any further growth (3 to 5 gm. of basal ration per day). The rats were maintained in this chronically starved condition for 3 weeks before sacrifice (Group 6).

The rations used for producing the biotin and pantothenic acid deficiencies were similar to those used in the riboflavin work, except that the liver filtrate was omitted and each rat received 40 γ of riboflavin daily. Biotin deficiency was produced by using 10 per cent egg albumin and 8 per cent casein in place of the usual 18 per cent casein, while the pantothenic acid deficiency was produced by omitting pantothenic acid from the basal ration. The animals were on the experimental diets for 9 to 11 weeks before sacrifice, at which time they exhibited the typical biotin and pantothenic acid deficiency syndromes.

Succinoxidase Determination—The succinoxidase content of freshly homogenized rat liver was determined according to the method of Pot-

average Q_{O_2} (cytochrome oxidase) value of 217 was observed in seven experiments on rats which were receiving the stock ration consisting of yellow corn, linseed oil meal, alfalfa leaf meal, crude casein, butter fat, calcium phosphate, sodium chloride, and whole liver powder. The cause of this disparity is not clear at the present, although it appears likely that the diet consumed may be the factor determining the content of cytochrome oxidase in liver tissue.

The results obtained with the use of the complete succinoxidase system (Column 4, Table I) are in good agreement with those of our previous experiments and lend further support to the belief that one or more components of the succinoxidase system other than calcium, aluminum, cytochrome *c*, and cytochrome oxidase are diminished in a riboflavin deficiency. As indicated from the results of Group 2b, excessive dosages of riboflavin are unable to increase the succinoxidase content of rat liver above the normal values.

Recent evidence by Swingle, Axelrod, and Elvehjem³ indicates that the stimulatory effect of calcium is due, at least in part, to its ability to stimulate the enzymatic destruction of coenzyme I, possibly through a nucleotidase system. The relative absence of coenzyme I prevents the conversion of malate to oxalacetate, which is a potent inhibitor of the succinoxidase system. Therefore, succinoxidase assays conducted in the absence of added calcium are apparently limited by the nucleotidase activity of the tissue.⁴ The results of such assays are given in Column 5 (Table I) and indicate that the nucleotidase activity of rat liver is not affected in riboflavin deficiency.

The results of studies in which a succinoxidase system rendered incomplete by the omission of cytochrome *c* is employed are given in Column 6. As previously stated, such assays are a measure of the cytochrome *c* content. It is apparent that there is no diminution in the liver cytochrome *c* content in riboflavin deficiency.

In the experiments reported in Column 7, both the cytochrome *c* content and the nucleotidase activity may be limiting factors. Since the nucleotidase activities do not vary with the different groups (see Column 5), the relative differences observed in Column 7 can probably be attributed to a slightly increased cytochrome *c* content in the riboflavin-deficient group.

Series II—The results of the experiments conducted in this series are given in Table II. Considering the variations in the liver weight to body

³ Swingle, K. F., Axelrod, A. E., and Elvehjem, C. A., unpublished experiments.

⁴ Other enzymes involved in the destruction of coenzyme I may be stimulated by calcium and the activities of these systems may be the actual limiting factors under these conditions.

weight ratios, it is evident that this ratio for the rats receiving therapy while on a restricted food intake (Group 3) is similar to that observed in the chronically starved rats (Group 6), while both of these values are considerably lower than those noted in deficient rats (Group 1) and in

TABLE II

Effects of Riboflavin Deficiency and of Chronic Starvation upon Glycogen, Fat, Protein, and Succinoxidase Activity of Rat Liver

Group No.*	No. of rats	Weight of rat	Liver	Glycogen†	Fat	Protein	Q _{O₂} ‡	Total succinoxidase activity of liver§
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Riboflavin deficiency								
1	12	52 (45-58)	6.0 (5.1-7.0)	4.5 (1.2-7.9)	3.2 (2.5-4.3)	19.9 (18.5-21.6)	63 (53-75)	57 (45-66)
2	7	179 (160-212)	4.6 (3.4-5.0)	6.4 (3.9-8.8)	3.5 (3.4-3.9)	18.9 (17.5-20.1)	90 (80-114)	
3	9	52 (47-57)	4.1 (3.7-5.5)	1.9 (0.02-4.5)	3.4 (2.4-4.5)	22 (18.2-25.2)	138 (107-168)	80 (68-89)
4	5	53 (44-66)	6.0 (4.2-7.0)	1.8 (0.2-3.0)	3.6 (3.7-5.4)	24 (21-26.3)	87 (78-111)	76 (72-83)
Chronic starvation experiment								
5	6	62 (53-69)	6.0 (5.5-6.5)	5.4 (3.9-6.4)	4.3 (2.6-6.3)	18 (17.3-18.6)	92 (86-106)	81 (75-85)
6	6	55 (54-57)	4.2 (4.0-4.6)	0.67 (0.03-1.8)	3.9 (3.3-4.8)	22.4 (20.6-23.0)	133 (125-145)	85 (77-90)

Average values are given. The range of values is given in parentheses.

* Group 1, basal ration; Group 2, basal ration plus 100 γ of riboflavin daily for 6 weeks, *ad libitum*; Group 3, basal ration plus 100 γ of riboflavin daily for 4 weeks, restricted food intake; Group 4, basal ration plus 100 γ of riboflavin daily for 3 days, restricted food intake; Group 5, normal rats; Group 6, normal rats chronically starved for 3 weeks.

† Glycogen, fat, and protein percentages are calculated on a fresh weight basis.

‡ Q_{O₂} values are those for the complete succinoxidase system and are expressed as c.mm. of oxygen consumed per mg. of dry weight per hour.

§ Q_{O₂} multiplied by the total dry weight of liver (gm.); calculated to a live body weight of 55 gm.

normal rats of this size (Group 5). This decrease in total liver weight observed in the restricted (Group 3) and the chronically starved rats (Group 6) is due to losses in glycogen, fat, and protein accompanied by corresponding changes in water content. No significant differences in the

moisture contents of the livers from the various groups have been noted. The ratio of liver weight to body weight for the animals receiving riboflavin therapy with *ad libitum* feeding (Group 2) is normal for male rats of this size, as determined in a large series of stock rats. A 3 day period of therapy with restriction of the food intake has no effect upon this ratio and an increase in total liver protein is actually observed (Group 4).

The Q_{O_2} values are in general agreement with those observed in previous experiments. The higher values noted in the restricted animals (Group 3) are most likely due to the fact that the period of riboflavin therapy was longer in this experiment than in the others.

Values obtained by multiplying the Q_{O_2} by the dry weight of the total liver have been taken as an arbitrary measure of the succinoxidase activity of the entire liver. Assuming that the liver weight varies in direct proportion to the body weight within the narrow body weight ranges obtained in these experiments,⁵ all of the liver weights in this series have been calculated for an original live body weight of 55 gm. This liver weight has been utilized in the calculations for the total succinoxidase activities given in Column 9 (Table II).

It is now evident that the interpretation of data obtained in controlled feeding experiments of this type is complicated by a number of factors. Animals receiving riboflavin therapy and maintained on a restricted food intake are in a chronically starved state, and this fact must be considered when Q_{O_2} data which have been expressed in the usual manner as oxygen consumption per mg. of dry tissue are evaluated. Thus the chronic starvation experiment clearly demonstrates that the Q_{O_2} values (Column 8) are markedly increased in the starved animals, while the succinoxidase content of the whole liver has not varied greatly. It is apparent that the losses in the liver constituents observed in chronic starvation are not paralleled by decreases in the succinoxidase content. In a similar manner, one may account for the relatively higher Q_{O_2} values observed in Group 3 (restricted for 4 weeks) as compared to those in Groups 2 and 4 in which starvation effects are excluded. Riboflavin therapy has resulted in an increase in the succinoxidase activity of the total liver (Column 9) and this fact is indicative of a relationship between riboflavin and the succinoxidase system.

The livers of nine biotin-deficient and six pantothenic acid-deficient rats were assayed for their succinoxidase activity. Average Q_{O_2} values of 89 and 83 were obtained in the biotin- and pantothenic acid-deficient rats, respectively. These values fall within the range observed in normal

⁵ Similar calculations have not been made for the animals in Group 2 (therapy with *ad libitum* feeding), since such a proportionality does not exist in a body weight range of 55 to 179 gm.

rats and indicate that the succinoxidase activity is unaffected in these deficiencies. Analyses of the liver to body weight ratios demonstrated the absence of a chronically starved state.

DISCUSSION

A complete description of the succinoxidase system is not possible at present. The available data indicate that succinate, succinic dehydrogenase, cytochrome *c*, cytochrome oxidase, and oxygen are obligatory components of this system. The function of calcium appears to be related to its stimulatory effect upon the enzymatic destruction of coenzyme I and the resulting inhibition of oxalacetate formation. The possibility that calcium functions also as a structural unit in the succinoxidase system is not entirely eliminated. Similarly, the recent experiments of Potter and DuBois⁶ indicate that aluminum may function not as a structural component of the succinoxidase system but as an agent neutralizing the toxic effect of an inhibitory compound, possibly copper. The existence of other components of the succinoxidase system has been indicated. Thus, cytochrome *b* (14) and a soluble protein activator (15) have been proposed as intermediary links in this system. Magnesium ions and the factors which are operative in the phosphate esterification which is coupled with succinate oxidation have also been suggested as possible additional factors (5, 16). A stimulatory effect of muscle juice has been observed by Ahlgren (17). Although the true significance of these factors cannot be assessed at present, they must be considered in the evaluation of the assay procedure for the succinoxidase system which is used in our experiments.

The data presented in this and in the previous experiment (1) establish that one or more components of the succinoxidase system other than cytochrome *c*, cytochrome oxidase, calcium, or aluminum are diminished in riboflavin deficiency. It may be deduced from these results that the component or components affected in the riboflavin deficiency are flavoproteins. Such a deduction gains support from the demonstrations that two flavoproteins *d*-amino acid oxidase and xanthine oxidase are diminished in riboflavin deficiency (18, 7). It may be that either succinic acid dehydrogenase itself is a flavoprotein or that another riboflavin compound is an intermediary cocatalyst serving as a hydrogen transport agent between succinate and the cytochrome system. A similar possibility has been suggested by Keilin and Hartree (14) as a result of their observation that the two absorption bands of a flavoprotein present in their succinoxidase preparation fade simultaneously with the appearance of the bands of reduced cytochrome when succinate is added. The probable production

⁶ Potter, V. R., and DuBois, K. P., unpublished experiments.

of H_2O_2 during the aerobic oxidation of succinate was noted by Lehmann and Mårtenson (19) and would appear to indicate the intermediation of a flavin compound. In such an event, the production of H_2O_2 would of necessity result from an incomplete oxidation of the reduced flavin intermediate by the cytochrome system. When the complex nature of the succinoxidase system is borne in mind, together with the difficulties attendant upon the determination of the exact constituent of this system which is being assayed in our procedure, it is apparent that final proof for the presence of a flavoprotein as a component of the succinoxidase system must await actual isolation. It is felt, however, that our data are very suggestive of such a possibility.

A point of interest has arisen from these experiments which bears upon the validity of enzymatic measurements expressed per unit of dry weight when conducted upon liver tissue from rats whose food intake has been restricted in controlled feeding experiments. Such paired feeding techniques are used extensively in nutritional work and were employed in our experiments to avoid effects inherently due to increased growth *per se*. However, complicating factors arise from the fact that the livers of chronically starved animals on a normal ration diminish markedly in their content of total dry matter, while the total succinoxidase content remains quite constant. In our experiments, the restriction of the food intake following riboflavin therapy results in the production of a chronically starved state which, in turn, leads to abnormally high Q_{O_2} values (oxygen consumption per mg. of dry weight). A similar effect of starvation upon the succinoxidase activity of rat liver has been noted by Rosenthal (20). The true change in enzyme content following riboflavin therapy can only be assessed by comparison of the total succinoxidase content of the livers from these restricted animals with that of the riboflavin-deficient group. The need for such comparisons when the size of an organ is a function of the nutritional state has been emphasized by Schultze (9). It is interesting to note that the percentage increase in the Q_{O_2} values following riboflavin therapy with *ad libitum* feeding is similar to the percentage increase in the total succinoxidase activity following riboflavin therapy on a restricted food intake (Table II).

The effect of the increased concentration of an enzyme per unit of weight of liver tissue upon the physiological efficiency of the enzyme remains an unanswered problem. An increase in physiological efficiency may have some bearing upon the validity of paired feeding techniques employed in nutritional studies.

SUMMARY

1. Further data have been offered in support of the premise that one or more components of the succinoxidase system are flavoproteins. Cyto-

chrome oxidase and aluminum have been eliminated as factors of the succinoxidase system which might possibly have been affected in riboflavin deficiency and thus complicate the assay for succinic dehydrogenase.

2. Cytochrome *c* and the enzyme systems involved in the destruction of coenzyme I are not diminished in riboflavin deficiency.

3. The succinoxidase activity of rat liver per unit of dry weight (Q_0) is increased in chronic starvation, while the total succinoxidase activity of the total liver is not affected. The importance of this factor in the evaluation of data obtained from controlled feeding experiments has been emphasized.

4. The succinoxidase activity of rat liver is not diminished in biotin and pantothenic acid deficiency.

BIBLIOGRAPHY

1. Axelrod, A. E., Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **142**, 85 (1942).
2. Potter, V. R., *J. Biol. Chem.*, **141**, 775 (1941).
3. Haas, E., Horecker, B. L., and Hogness, T. R., *J. Biol. Chem.*, **136**, 747 (1940).
4. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **140**, 931 (1941).
5. Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, **142**, 543 (1942).
6. Horecker, B. L., Stotz, E., and Hogness, T. R., *J. Biol. Chem.*, **128**, 251 (1939).
7. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **140**, 725 (1941).
8. Stotz, E., Sidwell, A. E., Jr., and Hogness, T. R., *J. Biol. Chem.*, **124**, 733 (1938).
9. Schultze, M. O., *J. Biol. Chem.*, **129**, 729 (1939).
10. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **106**, 485 (1933).
11. Channon, H. J., Platt, A. P., and Smith, J. A. B., *Biochem. J.*, **31**, 1736 (1937).
12. Johnson, M. J., *J. Biol. Chem.*, **137**, 575 (1941).
13. Stotz, E., *J. Biol. Chem.*, **131**, 555 (1939).
14. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **129**, 277 (1940).
15. Stern, K. G., and Melnick, J. L., *Nature*, **144**, 330 (1939).
16. Colowick, S. P., Welch, M. S., and Cori, C. F., *J. Biol. Chem.*, **133**, 359 (1940).
17. Ahlgren, G., *Skand. Arch. Physiol.*, **80**, 16 (1938).
18. Axelrod, A. E., Sober, H. A., and Elvehjem, C. A., *J. Biol. Chem.*, **134**, 749 (1940).
19. Lehmann, J., and Mårtensson, E., *Skand. Arch. Physiol.*, **75**, 61 (1937).
20. Rosenthal, O., *Biochem. J.*, **31**, 1710 (1937).

AUTOXIDATION OF STEROLS IN COLLOIDAL AQUEOUS SOLUTION

III. QUANTITATIVE STUDIES ON CHOLESTEROL

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The products formed by autoxidation of cholesterol in colloidal aqueous solution are 7-ketocholesterol, 7(α)-hydroxycholesterol, and 7(β)-hydroxycholesterol (1, 2). The last named compound was not identified as such, but in the form of a rearrangement product, Δ^6 -cholestenediol-3(β),5 (3). The $\Delta^{3,5}$ -cholestadienone-7 formed in small amounts when the reaction is carried out at elevated temperature is probably a secondary product.

This paper presents a study of the quantitative aspects of the autoxidation reaction under various conditions. The two main reaction products, the 7-ketone and the chromogenic diols, can be quantitatively differentiated in the crude autoxidized material by optical methods. The ketone shows strong selective light absorption at 240 $m\mu$, but since it gives no color with Lifschütz's reagent it does not interfere with the colorimetric determination of the diols; the latter in turn transmit light in the ultraviolet region, thus permitting the determination of the ketone by ultraviolet absorption measurements.

Since preliminary work had shown that temperature much more than any other variable determines the reaction rate, the reaction was carried out at either 85° or 37°, depending on the object of the experiment. It was found more convenient to investigate the influence of such factors as substrate concentration, pH, oxygen pressure, and nature of detergents in the fast reaction at 85°; at 37° the rate of autoxidation is very much slower, which renders this temperature more suitable for demonstrating catalytic effects.

EXPERIMENTAL

Methods

Preparation and Aeration of Colloidal Solutions—In preparing the colloidal solutions we adhered to the technique described in the first paper of this series (2), except for the following minor modifications. In most

* Recipient of a Squibb Research Fellowship, 1941-42, administered by the American Scandinavian Foundation. Present address, Caroline Institute, Stockholm, Sweden.

cases the sodium stearate was not added in substance, but in the form of a stock solution, which was prepared by dissolving 7.5 gm. of recrystallized stearic acid in 750 cc. of absolute alcohol, neutralizing with the required amount of standard alkali (26.3 mm), and making up with water to 1 liter. Before use the solution had to be slightly warmed to dissolve some crystalline soap which deposits on standing. Furthermore, in most runs a $M/15$ phosphate buffer solution (9 secondary : 1 primary) was added to stabilize the pH. The cholesterol solution was poured quickly into the rapidly stirred soap solution, which was previously heated to 85°. This moment was taken as the zero time when the subsequent aeration was carried out at that temperature. Whenever deviations from this procedure were necessary, this is indicated under "Results."

The solutions used in the autoxidation experiments at 37° were prepared in the same manner, usually in 1 liter batches, and rapidly cooled to approximately this temperature. No autoxidation products are formed during this short period, and control experiments in which the whole procedure was carried out in a nitrogen atmosphere showed that the presence of air during this period was immaterial for the course of the subsequent reaction at 37°. 50 cc. portions of the cooled solution were transferred to 125 cc. Erlenmeyer flasks, and the latter kept, loosely stoppered, in a water thermostat at 37°. The reaction rate at this temperature is so slow and the total oxygen uptake so small that diffusion from the atmosphere, aided by occasional shaking, apparently suffices to keep the concentration of active oxygen in the solution constant. Earlier control experiments in which larger volumes were aerated with oxygen at a slow rate had shown that in such solutions the formation of ketones at 37° was somewhat faster than in solutions merely kept at rest in the water bath, but the final values were the same. In view of this result, and also in order to avoid the marked and difficultly controllable increase in concentration caused by evaporation, the aeration was omitted in the routine procedure.

The pH was measured with a glass electrode on small samples withdrawn at the beginning of the reaction period. The solutions prepared by the above procedure usually had a pH of 9.0 to 9.5, and were adjusted with dilute hydrochloric acid to pH 8.0 to 8.5, which was the range preferred for most of the experiments.

Preparation of Samples for Measurements—Aliquots corresponding to 10 to 20 mg. of sterol were removed at intervals, acidified with a few drops of hydrochloric acid, and extracted with 15 cc. of ether. The ether solution was washed three times with water and evaporated to dryness on a steam-heated metal plate in weighed 25 cc. flasks under a stream of carbon dioxide. The weight of sterol in the residue was calculated

from the known proportion of sterol and stearic acid in the aqueous solution. The residue was dissolved in the calculated volume of absolute ethanol to give a concentration of 1 mg. of sterol per cc. Suitable aliquots of this solution were used for the spectrographic assay, and after replacement of the ethanol by chloroform, for the determination of the chromogens.

Colorimetric Determination of Chromogens by Lifschütz Reaction—Our method is a slight modification of that described by Blix and Löwenhielm (4). Instead of the Hüfner spectrophotometer we used a Zeiss stufenphotometer with Filter S-62. Our standard substance was pure 7(β)-hydroxycholesterol containing 1 mole of methanol of crystallization (5) instead of the ill defined non-crystalline "oxycholesterol" used by Blix and Löwenhielm. The color reagent was prepared by dissolving 0.1 gm. of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 90 cc. of glacial acetic acid and then adding 10 cc. of concentrated sulfuric acid. When kept in a well stoppered bottle, it is stable for at least a month.

The sample is dissolved in 1.5 cc. of pure chloroform containing 1 per cent ethanol. On addition of 3 cc. of the reagent a bluish green color develops immediately, which soon turns pure blue. It reaches its maximum intensity in 5 minutes and then remains constant for 15 to 20 minutes, provided that the solution is kept in a well stoppered flask.

As can be seen from the standardization curve of 7(β)-hydroxycholesterol (Fig. 1), the slope of the curve increases with concentration. This was the case also with all other pure chromogens tested. The results are reproducible within about ± 5 per cent.

In view of the complex nature of the autoxidized mixture, and of the statement of Blix and Löwenhielm that soaps interfere with the color reaction, the influence of cholesterol and 7-ketocholesterol and of fatty acids was investigated over the whole range of concentration. The fatty acids (palmitic and stearic acid) were added in amounts equal to that of the standard substance at each concentration (0.1 to 0.5 mg.), while the amount of sterol added (4 mg.) was the same at each concentration of the standard. The curves thus obtained showed no deviation from the standard curve.

Since 7(α)-hydroxycholesterol is one of the autoxidation products, the curves for this substance and of Δ^6 -cholestenediol-3(β),5, a rearrangement product of 7(β)-hydroxycholesterol (3), were determined. Similar measurements were carried out with the benzoyl derivatives of these three chromogens and, in view of the possible usefulness of the method in related problems, with a few other sterols known to give the Lifschütz reaction. Most of these curves, when plotted for equimolecular amounts, fall fairly close to the curve of 7(β)-hydroxycholesterol. In order to indicate their approximate locations the D values for 2.5, 5.0, 7.5, and 10×10^{-4} mm are

given in Table I. The values for the two epimeric 7-hydroxycholesterols and their mono- and dibenzoates all fall in a range which is not much greater than the error of the method. It therefore appears that the spatial orientation of the 7-hydroxy group and esterification of this and of the 3-hydroxy group exert comparatively little influence. These findings not only justify our practice, in this and in our previous work, of expressing the *total* chromogen content of the autoxidation products in terms of the 7(β) epimer, but they also show that no appreciable error is introduced when the method is applied to esterified fractions. The nature of the side chain is of little importance, but constitutional factors residing in

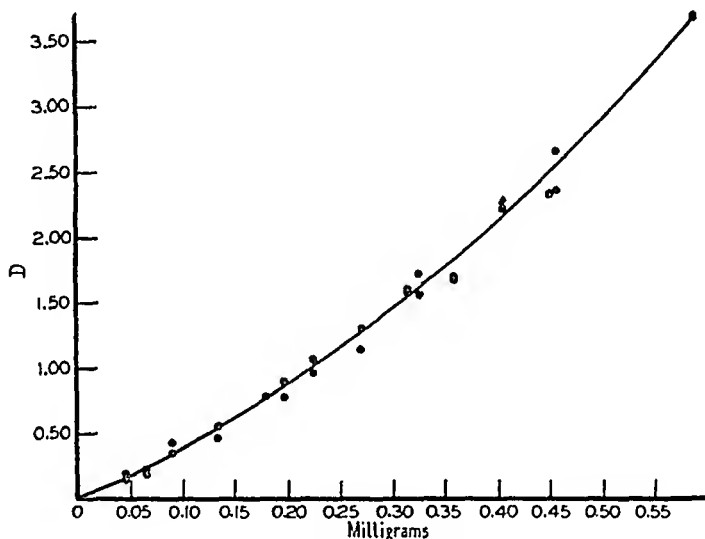


FIG. 1. Standardization curve for 7(β)-hydroxycholesterol. Ordinate unit, $D = \log I_0/I$, for 1 cm. cell length. The color was developed as described in the text from the amounts indicated on the abscissa.

Rings A and B markedly influence the chromogenic potency. Δ^6 -Cholestenediol-3,5 is considerably more, and Δ^6 -cholestenediol-3,4 very much less, chromogenic than the 3,7-diols. 7-Dehydrocholesterol benzoate, contrary to expectation, is a relatively weak chromogen. It is therefore improbable that the chromogen formation from the 3,7-diols involves the intermediary production of 7-dehydrosterols. The high chromogenic power of Δ^6 -cholestenediol-3(β),5 suggests that a $\Delta^{2,4,6}$ -triene, or $\Delta^{4,6}$ -cholestadienol-3, may be the ultimate precursor of the blue pigment. Allylic shifts (Δ^6 -3,7-diols \rightarrow Δ^6 -3,5-diols) and subsequent dehydration to either of the compounds mentioned could easily occur under the influence of the strongly acidic medium in which the color reaction is carried out.

Spectrographic Determination of 7-Ketocholesterol—The ketone in the aliquot samples was determined by measuring the optical density at 240 m μ in a Hilger spectrophotometer. 7-Keto- $\Delta^{3,5}$ -cholestadiene, which is formed in small amounts in the reaction at 85°, does not absorb light in this region. The calculation of the yields was based on the previously determined (2) molecular extinction coefficient 12,500 for the pure ketone. The densities in the measurements were spaced in such a way that the error in determining d_{\max} was not greater than ± 5 per cent.

TABLE I

Chromogenic Potency of Various Sterols and Sterol Esters in the Lifschütz Reaction

D as defined in Fig. 1. The D values were obtained by interpolation from smooth standardization curves which were determined as that in Fig. 1, but were plotted in moles instead of mg. as abscissa units.

Compound	$D, 1 \text{ cm.}$			
	2.5 mm $\times 10^{-4}$	5.0 mm $\times 10^{-4}$	7.5 mm $\times 10^{-4}$	10 mm $\times 10^{-4}$
7(β)-Hydroxycholesterol	0.36	0.84	1.40	2.04
“ dibenzoate	0.34	0.77	1.32	1.95
7(β)-Hydroxycholesterol-7-monobenzoate*	0.39	0.90	1.53	2.24
7(α)-Hydroxycholesterol* ...	0.38	0.86	1.48	2.15
“ dibenzoate	0.34	0.80	1.36	2.00
7(α)-Hydroxycholesterol-7-monobenzoate (cf. (6))	0.41	0.92	1.58	
7-Dihydrocholesterol benzoate	0.21	0.49	0.75	1.02
Δ^4 -Cholestenediol-3,5	0.47	1.16	2.02	3.05
Δ^4 -Cholestenediol-3,5-monobenzoate	0.36	1.00	1.68	2.45
Δ^5 -Cholestenediol-3,4	0.05	0.14	0.23	0.33
7(α)-Hydroxystigmasterol dibenzoate	0.30	0.72	1.21	1.76
7(α)-Hydroxystigmasterol-7-monobenzoate (cf. (6))...	0.35	0.86	1.49	2.19

* Wintersteiner, O., and Ruigh, W. L., *J. Am. Chem. Soc.*, in press.

Results

In order to simplify the calculation of the yields of ketone and of chromogens¹ no correction was made for the change in molecular weight due to their uptake of oxygen. These corrections (-3.5 and -4.0 per cent respectively of the yields given) are much smaller than those for the over-all error in a single determination and would not affect the general conclusions drawn from the experiments.

Reaction Temperature 85° Fig. 2 shows the results of two typical experiments; one of these, Experiment 1, exemplifies our “standard conditions,” except that air was subsequently used instead of oxygen.

¹ Ketone and chromogen are denoted by Ke and Ch, respectively, in this paper.

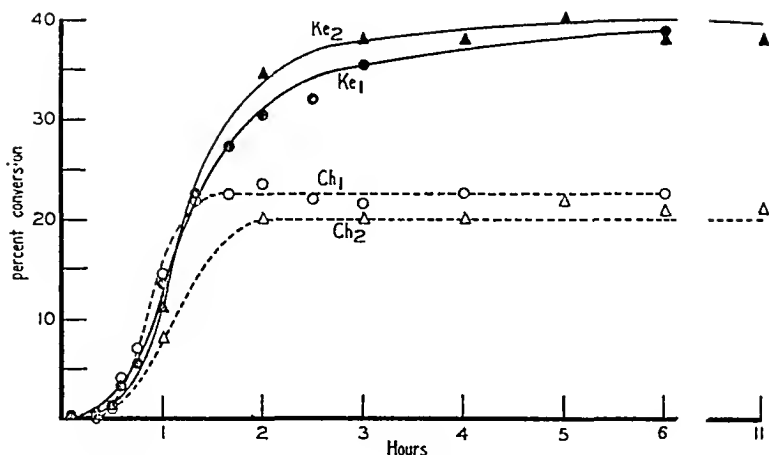


FIG. 2. Autoxidation of cholesterol at 85°. Experiment 1, 1 gm. of cholesterol in 50 cc. of alcohol; 30 cc. of sodium stearate, 10 cc. of phosphate buffer, 1 liter of water; pH 8.6; oxygen. Experiment 2, 500 mg. of cholesterol in 50 cc. of alcohol; 20 cc. of sodium stearate, 1 liter of water; pH 8.3; oxygen. Experiment 1, solid lines, ketone (Ke₁) solid circles, chromogen (Ch₁) open circles; Experiment 2, broken lines, ketone (Ke₂) solid triangles, chromogen (Ch₂) open triangles.

Experiment 3—In this experiment the conditions were similar to those employed in our qualitative studies (2): 5 gm. of cholesterol in 200 cc. of alcohol; 0.5 gm. of stearate in substance, 10 cc. of phosphate buffer, 1 liter of water; pH 8.3; 85°; air. The reaction curves closely resembled those in Experiments 1 and 2; after 3 hours the values of Ke and Ch became constant at 44 and 22 per cent respectively.

The curves clearly show that the formation of Ch ceases after 1 to 2 hours, and that of Ke after 3 to 4 hours, leaving a considerable portion of the cholesterol unattacked. This inhibition effect is typical of all experiments carried out at 85°, though the time relations vary somewhat with the conditions. The final levels of Ke and Ch were occasionally higher than those shown in the curves (45 and 25 per cent respectively). Furthermore, the length of the induction period seen in the curves is subject to considerable variation even under apparently identical conditions. In some experiments carried out under "standard conditions" no induction period at all was noted; the reaction proceeded from zero time at an approximately constant rate, and the time necessary to reach the level state was correspondingly shorter. It is likely that these variations depend largely on the initial degree of dispersion, or on other difficultly controllable factors such as the presence of minute but varying amounts of catalysts in the substrate and the reagents. At any rate, the final state

reached by the system seems to be largely independent of the somewhat variable course of the reaction in the initial phase.

It furthermore is evident from Experiments 1, 2, and 3 that variation of the oxygen pressure between 1 and 0.2 atmosphere, and of substrate concentration between 0.05 and 0.5 per cent, does not modify the results.

Influence of pH and of Detergent—On account of the decreased emulsifying power of stearate at pH values lower than 7 the influence of pH was investigated with Duponol PC as the detergent. In these experiments (Nos. 4 to 7) some of the analytical samples were lost, as they became dark brown on evaporation of the ether, probably because the laurylsulfonic

TABLE II
Influence of pH and Detergent

Experiment 4—500 mg. of cholesterol in 60 cc. of alcohol; 1 gm. of Duponol, 10 cc. of phosphate buffer in 1 liter of water. *Experiment 5*—As in Experiment 4, but 30 mg. of cholic acid, neutralized with sodium hydroxide, instead of Duponol. *Experiments 6 and 7*—As in Experiment 4, except for the pH adjustment. Reaction temperature 85°.

Ex- per- iment No.	Detergent	pH		0.5 hr	1.0 hr.	1.5 hrs.	2 hrs.	2.5 hrs	3 hrs.	5 hrs.	6 hrs	9 hrs.
				per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
4	Duponol	7.8	Ke	0	Trace	4.0	9	14		41		
			Ch	0.5	1.4	2.1	10	22		18		
5	Na cholate	7.8	Ke	1.9	4.5		18		40	38		
			Ch	0	Trace		11		21	18		
6	Duponol	6.5	Ke		0.6		6.4		13	35		45
			Ch		Trace		6.0		9	19		17
7	"	9.5	Ke	3.7	8.0				38		43	
			Ch	3.0	7.0				19		20	

acid (Duponol) had not been completely removed. Sodium cholate was used in one experiment in which the pH was near the usual range.

The final levels of Ke and Ch in the four experiments recorded in Table II are not markedly different from those obtained under "standard conditions." In graphic representation a prolongation of the short induction period seen in Fig. 2 is apparent at pH 7.8 with Duponol and with cholate. At pH 6.5 with Duponol this delay is even somewhat greater, while at pH 9.5 with the same detergent the reaction seems to start faster than under "standard conditions." All in all the effect of pH on reaction rate within the range 6.5 to 9.5 was not of a magnitude to warrant more detailed examination. Also, in view of the facts pointed out in the preceding section, it is questionable whether the observed differences in the induction period are solely referable to the variation of the pH.

Other experiments at 85° were carried out mainly to ascertain the reason for the incompleteness of the autoxidation.

Experiment 8. Standard Conditions—The final levels of Ke and Ch were 40 and 20 per cent respectively. The aeration was interrupted after 5 hours and the sterol mixture was isolated as previously described (2). The dried material was then reemulsified with stearate and aerated in the usual manner for several hours. Contrary to our expectation the above levels of Ke and Ch remained absolutely unchanged throughout this period. A similar experiment with stigmasterol, which is recorded in detail in Paper IV of this series, confirmed this result in its essential point. This finding renders it improbable that the inertness of the remaining cholesterol towards further attack by oxygen is due to insufficient emulsification of a portion of the original substrate, or to a change in the degree of dispersion during the reaction. The factor concerned must be either (1) an inhibiting substance formed during the reaction, or (2) a preformed catalyst which loses its effectiveness as the reaction progresses. In view of the outcome of Experiment 8 it is unlikely that such substances are derived from the soap or from the solvents. That the emulsifying agent plays a rôle in limiting the reaction is also improbable in view of the fact that the same degree of inhibition was observed with such chemically dissimilar detergents as stearate, cholate, and Duponol. All the facts therefore point to the substrate or to the reaction products as the ultimate source of the inhibition.

To decide between the two alternatives mentioned, a synthetic mixture simulating the composition of the autoxidized material was emulsified and aerated (Experiment 9, Table III). All four compounds used were prepared by the usual chemical methods and not by autoxidation in order to exclude the presence of traces of yet unidentified autoxidation products, which might act as inhibitors. The Ke and Ch levels remained exactly at the initial values. This result proves that the inhibition is caused by the accumulation of the reaction products and not by inactivation of a catalyst derived from cholesterol.

In the following experiments (Nos. 10 to 12) each of the reaction products was tested separately for its inhibiting power, except for the two diols which were added together. The initial percentage of these compounds in the mixture corresponded to their final level under standard conditions; the remainder was made up of cholesterol. The presence of 40 per cent ketone (Experiment 10) permits the production of the usual amount of chromogens, but markedly inhibits ketone formation. A deficit is apparent in the determination of the initial Ke value (35 instead of 40 per cent); so that the calculated percentage of newly formed ketone (15.7) is probably somewhat too high. On the addition of a small amount of 7-keto-

cholestadiene (Experiment 11) the formation of 7-ketocholesterol is also markedly, and that of chromogens slightly, inhibited. Conversely, the presence of 20 per cent of a mixture of the chromogenic diols (Experiment 12) decreases chromogen production to about one-third of the expected value, while the percentage of newly formed ketone is proportionally greater, though it also falls short of the usual 40 per cent. Although those figures in the last column of Table III which are computed from differences in initial and final levels are necessarily subject to considerable error, two significant facts are clearly revealed by the results. The extent

TABLE III

Autoxidation of Cholesterol at 85°, with Reaction Products Added at Start

Mixtures, about 100 mg., in 5 cc. of alcohol; 3 cc. of sodium stearate, 0.9 cc. of phosphate buffer, 100 cc. of water. I, cholesterol; II, 7-ketocholesterol; III, 7(α)-hydroxycholesterol; IV, 7(β)-hydroxycholesterol; V, 7-keto- $\Delta^{3,5}$ -cholestadiene.

Experiment No.	Composition mg.	pH		0 hr.	1 hr.	2 hrs.	3 hrs.
				per cent	per cent	per cent	per cent
9	40.3 I, 39.8 II, 10.1 III, 9.8 IV, 3.1 V	8.0	Ke	42	42	42	42 (0)
			Ch	19.0	18.8	16.7	18.7 (0)
10	61.1 I, 39.5 II	8.0	Ke	35	38	42	45 (15.7)
			Ch	0	7.7	9.5	11.4 (18.8)
11	94.5 I, 5.5 V	8.2	Ke	1.3	9.6	12.8	12.8 (13.5)
			Ch	0	10.7	14.3	13.8 (14.6)
12	80.4 I, 9.9 III, 10.2 IV	7.9	Ke	0	3.0	19.2	19.2 (24.0)
			Ch	18.8	20.5	24.6	24.1 (6.6)

The figures under "per cent" designate the amounts of ketone and chromogen present in the mixture after 0, 1, 2, and 3 hours, expressed as per cent of total sterol. The figures in parentheses in the last column designate ketone and chromogen newly formed after 3 hours from the cholesterol initially present, expressed as per cent of cholesterol.

$$\text{Thus (Ke)} = \frac{(\text{Ke}_{3 \text{ hrs.}} - \text{Ke}_0)100}{\% \text{ cholesterol}}$$

of total autoxidation is markedly decreased by any single one of the reaction products, and the inhibition is more specifically, though not exclusively, directed against the formation of those end-products which are of the same type as the inhibitor; i.e., either 7-ketones or 7-hydroxy compounds. It is obviously the summation of these inhibiting effects which brings the reaction as a whole to a standstill.

A few experiments were performed at 85° to supplement our observations on the effect of certain solvents at 37°. The addition of 2 cc. of pyridine per liter, which increased the speed and extent of the formation of Ke over the control at 37°, had no such effect at the higher temperature.

However, the substitution of acetone for alcohol as the solvent for cholesterol completely prevented the reaction, as it did at 37°. Since alcohol was added with the soap, this inhibition was not due to absence of this solvent.

Reaction Temperature 37°. Fig. 3, Experiment 13—Fig. 3 shows the course of the reaction at 37°. It will be noted that the abscissa units are days instead of hours as in Fig. 2. The yield of Ke in this experiment reached 65 per cent after 12 days, while the formation of Ch ceased after 2 days at a level of 10 per cent.

Experiment 14—0.5 gm. of cholesterol in 50 cc. of alcohol; 20 cc. of sodium stearate, 20 cc. of buffer in 1 liter of water; pH 8.4; after 10 minutes stirring under N₂ at 85° and cooling three aliquots were taken. Solution A,

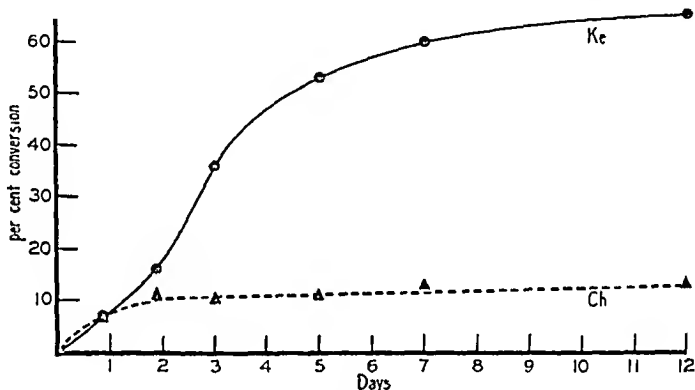


FIG. 3. Autoxidation of cholesterol at 37°. Experiment 13, 100 cc. portion of colloidal solution prepared at 85° from 5 gm. of cholesterol, 150 cc. of alcohol, 1 gm. of sodium stearate, 10 cc. of phosphate buffer, 1 liter of water; pH 8.0; reaction temperature 37°.

500 cc., 37°, aeration with O₂ (slow bubbling); Solution B, 200 cc., cooled in ice and kept at about 4° in the refrigerator; Solution C, 125 cc., saturated with O₂ and kept at room temperature (about 25°). The Ch curve obtained for Solution A was similar to that of Experiment 13, the level becoming established at 11 per cent after 2 days, and remaining approximately constant up to 18 days (13 per cent). The curve for Ke differed from that in Experiment 13 in that the level (64 per cent) was reached after 4 days and then remained unchanged up to 18 days. No Ke and Ch were formed in Solution B up to 18 days. Solution C, 2 days, Ke 26 per cent; 18 days, Ch 14 per cent, Ke 60 per cent.

It appears from Experiments 13 and 14 that at 37° the production of Ke is considerably greater, and that of Ch lower, than at 85°. However, it

must be emphasized that the yields, especially of Ke, are not as well reproducible as those obtained at the higher temperature. Though the high Ke values recorded above could be duplicated in a few more experiments, in others they were considerably lower (40 per cent or even less). Also, the shape of the curves, especially in the initial phase up to 4 days, varies somewhat. As far as the results obtained with the present procedure permit any conclusions, it appears that one of the factors causing variable results is the initial degree of dispersion, which probably cannot be exactly reproduced in every instance. Another factor may be the formation, during the short heating required in the preparation of the sols, of small but variable amounts of autocatalytic substances from the substrate. In the following experiments designed to test the influence of various additions the same colloidal solution, prepared in bulk at 85°, was therefore used for as many simultaneous experiments as practicable, and an aliquot without any addition ("blank") was always run alongside the experimental solutions in order to establish a basis for comparison.

Influence of Cyanide and of Heavy Metals—In Experiment 14 a 125 cc. aliquot containing 0.2 mg. of sodium cyanide per cc. (4×10^{-3} M) was treated in the same way as Solution A. No Ke or Ch was found to be present after 18 days. The effect of smaller additions of cyanide was tested in Experiment 15 (Table IV).

The reaction was still completely inhibited after 9 days when sodium cyanide was present in a concentration of 0.8 γ per cc. (1.6×10^{-5} M). With 3.2×10^{-6} M of cyanide no reaction had occurred up to the 6th day, but within the next 6 days the oxidation had started and reached the blank levels on the 12th day. As the concentration of cyanide was further reduced, the inhibition period became shorter, and with 5×10^{-8} M the reaction approximated that in the blank. The addition of cupric ions in equimolecular amounts shortened the inhibition period (Solution 10), but a 4-fold excess was apparently no more effective, the reaction being still held in abeyance after 2 days (Solution 11). However, with the same molecular ratio (5:1) the addition of cupric ions to a solution inactivated by cyanide for 3 days immediately started the reaction at a rate at least as fast as in the blank experiment. The reactivation by cupric ions, as well as the immediate stopping of the reaction by cyanide after it has been in progress for some time, is illustrated in Fig. 4 (Experiment 15a).

The addition of potassium ferrocyanide (Experiment 15, Solution 13) caused a partial inhibition, but the effect is very much less pronounced than with cyanide in comparable concentration.

Cupric ions in comparatively high concentration did not increase the rate and final levels seen in the blank (Experiment 15, Solution 12). The effect of other heavy metals is recorded in Table V, Experiment 16. The oxida-

TABLE IV

Influence of Cyanide and Heavy Metals (37°)

Experiment 15—50 cc. portions of colloidal solution prepared at 85° from 1 gm. of cholesterol, 50 cc. of alcohol, 30 cc. of sodium stearate, 10 cc. of buffer, 1 liter of water; pH 8.2; reaction temperature 37°.

Solution No.	Additions	2 days		4 days		6 days		12 days	
		Ke	Ch	Ke	Ch	Ke	Ch	Ke	Ch
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
1	None (blank)	32		35	21	42	21	42	15
2	NaCN 2×10^{-3} M	0	0	0	0	0	0	0*	0*
3	" 8×10^{-5} "	0	0					0*	0*
4	" 1.6×10^{-5} M	0	0					0*	0*
5	" 3.2×10^{-6} "	0	0	0	0	0	0	42	19
6	" 8×10^{-7} M	0	0	0	0	19	17	42	18
7	" 2×10^{-7} "	11		32	20	42	22	42	16
8	" 5×10^{-8} "	19	19	32		42	20		
9	" 1.25×10^{-8} M	22	21	39	21	42	22		
10	" 3.2×10^{-6} " CuSO ₄	0	0			19	16	42	18
	3.2×10^{-6} M								
11	NaCN 3.2×10^{-6} M, CuSO ₄	0	0			21	17	42	
	1.6×10^{-6} M								
12	1 mg. CuSO ₄ (8×10^{-5} M)	32	23	39	21	42	21	42	15
13	8.4 mg. K ₄ Fe(CN) ₆ (4×10^{-4} M)	3	6.5	7	12				19

* 9 day values.

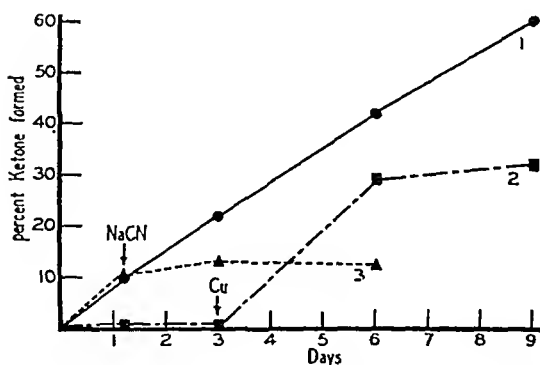


FIG. 4. Inhibition of autoxidation by cyanide and reactivation by cupric ions. Experiment 15a, 49 cc. portions of colloidal solution prepared as in Experiment 15 (Table IV). Curve 1, blank (49 cc. + 1 cc. of water); Curve 2, 49 cc. + 0.2 mg. of NaCN in 1 cc. of water (8×10^{-5} M); after 3 days 5 mg. of CuSO₄·5H₂O in 1 cc. of water were added; Curve 3, 49 cc.; after 28 hours 1 mg. of NaCN in 1 cc. of water (4×10^{-4} M) was added. pH 8.3; reaction temperature 37°.

tion was accelerated in the initial stage by ferrous ion, and even more so by zinc ion, but became stabilized at the blank levels. Mercuric ion, on the other hand, retarded the speed of the reaction. A very marked inhibition which persisted for at least 6 days was caused by manganese II ion. A similar effect of manganese was observed in an experiment carried out at 85°; in this case no reaction at all occurred during the experimental period (5 hours).

Table V also contains an experiment (Solution 2) which shows that there is no perceptible difference in the rate and extent of the reaction when the pH is lowered from 8.2 to 7.4.

TABLE V
Influence of Heavy Metals

Experiment 16—1 liter of colloidal solution prepared at 85° as in Experiment 15 (Table IV), except that all operations were carried out under nitrogen till after cooling. The 50 cc. portions were aerated shortly for saturation with air oxygen. pH 8.2, reaction temperature 37°.

Solution No.	Additions	2 days		4 days		6 days		10 days	
		Ke	Ch	Ke	Ch	Ke	Ch	Ke	Ch
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	None (blank)	14.4	12.5	38	16	45	16	48	14
2	" " pH 7.4	14.4	12.5	38	15	45	16	48	15
3	2 mg. FeSO ₄ ·7H ₂ O (1.4 × 10 ⁻⁴ M)	24	13	42	14	38	13	45	15
4	2 mg. ZnCl ₂ (3 × 10 ⁻⁴ M)	35	15	42	14	42	14	42	12
5	2 " HgCl ₂ (1.5 × 10 ⁻⁴ M)	11.2	8.8	22	10.5	38	14	45	15
6	2 " MnCl ₂ ·4H ₂ O (2 × 10 ⁻⁴ M)	0.96	2.2	0.96	2.4	4.2	6.0	26	12

Other Additions—Phenol (10 mg. per 50 cc.), which is known to form complexes with heavy metals, prevented the reaction completely up to 12 days. The same result was obtained with salicylaldehyde (10 mg. per 50 cc.), which more specifically inhibits catalysis by copper, and, contrary to expectation, with hemin (1 mg. per 50 cc.). The latter compound was dissolved in pyridine before addition, and consequently a control with the same amount of pyridine alone (0.1 cc. per 50 cc.) was set up. In this solution the reaction proceeded much faster, and more ketone was formed than in the blank. The values obtained after 2 and 10 days will suffice to illustrate this effect.

Blank. 2 days, Ke 5.7, Ch 9.0; 10 days, Ke 28, Ch 14

Pyridine. 2 " " 42, " 14; 10 " " 64, " 13.5

The experiment was repeated with additions of 0.1 and 0.01 cc. of pyridine per 50 cc., and spacing of the determinations within the first 4 days. The accelerating effect was confirmed in both cases. Only the final (4 day) values of K_e are given here: blank, 29; pyridine additions, 48 and 50 respectively. On the other hand, the addition of collidine (0.1 cc. per 50 cc.) gave values little different from those of the blank.

In another set of experiments the influence of organic solvents was investigated. The usual course of the reaction was not altered by more than doubling the amount of alcohol over that added with the soap and cholesterol. The presence of 10 per cent methanol or dioxane in addition to the ethanol had no influence, while acetone in the same concentration caused complete inhibition. With 4 per cent acetone the inhibition was only partial.

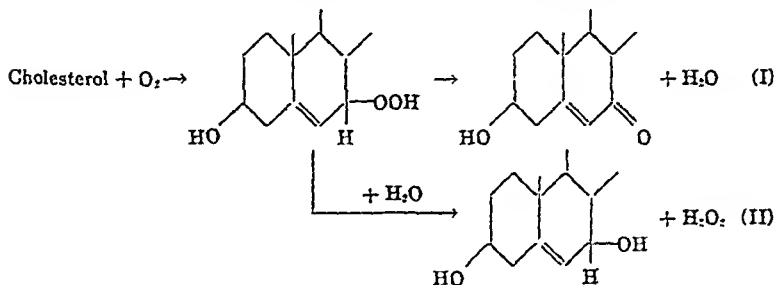
DISCUSSION

The type of autoxidation which cholesterol undergoes, namely attack of the methylene group next to the double bond leading to the formation of the corresponding α , β -unsaturated ketone and alcohol, has numerous parallels in the cyclohexene and terpene series. Thus α -pinene is slowly oxidized at room temperature by air oxygen to verbenone and verbenol (7, 8), and limonene to carvone and carveol (9); in the case of β -phellandrene only the ketone has been isolated (10), while cyclohexene seems to yield preponderantly Δ^2 -cyclohexenol-1 (11-13). These reactions are effectively catalyzed by colloidal osmium (8, 14) and, as Cook's (13) more recent work shows, by iron phthalocyanine. The latter study is of particular interest here, because cholesterol was among the substrates used. While the compounds containing the cyclohexene nucleus, and others such as tetralin, Δ^2 -octalin, and diphenylmethane were readily autoxidized in the presence of the catalyst, cholesterol in benzene solution failed to take up any oxygen even after several hours at 80°. Cholesteryl acetate, on the other hand, when similarly treated in xylene for 42 hours, yielded some 7-ketocholesteryl acetate besides unidentified by-products. It is interesting to note that in aqueous colloidal solution the situation is just the opposite. Here a free hydroxyl group is a prerequisite for rapid oxidation, while esterification all but prevents the reaction (Paper IV of this series). Cook's view that the activating effect of the double bond is counteracted by the free 3-hydroxy group is therefore too simple an explanation which does not cover all the facts. What seems to matter in both cases is the polarity of the substrate relative to that of the solvent, a relationship which in some way must determine the manner in which Ring B is exposed to, or brought in contact with, the catalyst and with molecular oxygen.

Cook's experiments as well as some of the previous work on terpenes

indicate that autoxidations of this type are markedly accelerated by, if not wholly dependent on, heavy metal catalysis. In the case of colloidal cholesterol the participation of heavy metals seems to be obligatory, as the system is inhibited by extremely small amounts of cyanide, but can be reactivated by an excess of cupric ions. Since the spontaneous reaction is somewhat, but not greatly accelerated by the addition of zinc or ferrous ions, the traces of heavy metals present in the substrate or the reagents must be nearly equal to the catalytically optimal amount. It seems probable that the much greater ease with which cholesterol is autoxidized in the colloidal than in the molecular dispersed or molten state is due to the fact that the substrate itself may act as the carrier for the catalyst and thus may increase the efficiency of the latter.

There is hardly any doubt that this catalysis is concerned with the formation or decomposition of an unstable cholesterol peroxide, or most probably with both these reactions. Stable moloxides or peroxides of cyclohexene



(15) and tetralin (16) are known to exist, and tetralin peroxide on addition of iron phthalocyanine rapidly decomposes to α -tetralone and water (13). In the case of cholesterol such a mechanism would readily account for the formation of the 7-ketone (Equation I). The 7-hydroxy compounds could arise by interaction of the unstable peroxide² with water with the elimination of hydrogen peroxide, the latter being subsequently decomposed catalytically by the heavy metal (Equation II).

A 2-step reaction, in which the diols formed in Equation II are subsequently oxidized to the ketone, appears out of the question, because these compounds remain entirely unchanged on aeration in colloidal solution.

² The formulation of the unstable cholesterol peroxide as in Equation I (α -methylenic hydroperoxide type, as opposed to the double bond adduct type) is in accord with the conclusion reached by Criegee *et al.* (18), and more recently by Farmer and Sundralingam (19) regarding the structure of cyclohexene peroxide. An analogous formulation has also been adopted by Cook (13) for tetralin peroxide.

As to the reverse reaction, the only way by which the diols could arise from the ketone is by interaction of the latter with cholesterol and water, which is highly improbable.

The fact that both epimeric 7-hydroxycholesterols are formed requires some comment. The 7(β)-diol has hitherto been accessible only by permanganate oxidation of cholesteryl acid phthalate, but we have recently found that it can also be obtained, together with the α epimer, by reduction of 7-ketocholesterol with aluminum isopropylate.³ However, as pointed out above, it is difficult to fit such a reductive mechanism into the autoxidation scheme. We prefer the simpler explanation that in the fixation of oxygen at C₇ either of the 2 hydrogen atoms may be replaced by the peroxidic group, and that the configurations thus established persist when the stereoisomeric peroxides are decomposed to the diols.

In general it does not seem likely that the formation of Ke and Ch, though it always occurs simultaneously, is linked in a coupled reaction. The fact that all extraneous inhibitors so far discovered (cyanide, manganese, phenol, salicylaldehyde, hemin, acetone) block both these pathways is hardly evidence to the contrary, since they may do so simply by preventing the formation of the common precursor. Furthermore, the partial inhibition set up by each of the reaction products, which is specifically directed against the formation of compounds of the same type as the inhibitor, indicates that the two pathways are at least to a certain extent independent. However, since in spite of the inhibited formation of one type of end-product, for instance of Ch, the other, Ke, is not proportionally increased, it is necessary to assume that the inhibition set up by the end-products extends to the formation of the precursor. The existence of true equilibria, such as these experiments suggest, is difficult to reconcile with the generally irreversible nature of autoxidative processes in which peroxides are intermediates, as well as with the older views of colloid chemistry which consider colloidal systems as essentially "diphasic." The latter objection does not necessarily apply if the sols used by us represent partially or completely "solubilized" systems, as defined by McBain (17).

Some of the results obtained with catalysts and inhibitors might be considered at this point. Ferrous and zinc ions, though they accelerate autoxidation in the initial phase, do not increase the yields of Ke and Ch beyond those attained in the spontaneous reaction. When the onset of the reaction is delayed, as is the case with intermediate concentrations of cyanide (Table IV, Solutions 5 to 7), the levels eventually reached and maintained are likewise those of the blank experiment. After this state is once reached, the addition of a heavy metal catalyst does not produce any change. Thus the system tends to approach a steady state which resembles an equilibrium

³ Wintersteiner, O., and Ruigh, W. L., *J. Am. Chem. Soc.*, in press.

in so far as it is independent of the rate by which it is attained. On the other hand, it is difficult to conceive that the over-all reaction is in any sense reversible. Most likely the phenomenon is somehow connected with the colloidal state of the system. Since it was shown that insufficient dispersion of a part of the substrate cannot be the limiting factor, one might think of a gradual displacement of the substrate on the surface of the micelle by the more polar end-products, preventing further fixation of oxygen by the former. However, this concept would require some additional hypothesis to explain the specific inhibition effects exerted by the single reaction products.

This as well as various other aspects of the present study will have to be clarified by future work. Especially the paradoxical finding that hemin blocks the autoxidation invites closer examination of the rôle played by heavy metal complexes in general. Another of our objects is to approximate physiological conditions still more closely by eliminating the organic solvent and the detergent. Qualitative evidence (4) indicates that these components are not essential for the autoxidation of colloidal cholesterol. It also remains to be determined how the reaction proceeds in biological media and in the presence of biocatalysts.

SUMMARY

The autoxidative formation of 7-ketocholesterol and of 7-hydroxycholesteroles from aqueous cholesterol sols has been studied quantitatively under various conditions. The speed of the reaction is primarily dependent on temperature, while concentration, pH, oxygen pressure, and the nature of the detergent exert comparatively little influence. At 85° the reaction invariably comes to a standstill after a few hours with about 40 per cent of ketone and 20 per cent of the diols formed. It has been shown that it is the accumulation of the reaction products which limits autoxidation to these levels. Both types of reaction products participate in bringing about this inhibition, but each of them more specifically hinders the formation of its own kind.

Small quantities of cyanide completely stop the autoxidation. With still lower concentrations of cyanide the reaction is merely delayed, and then proceeds till the normal levels are reached. The cyanide-inhibited system can be reactivated by cupric ions. Ferrous and zinc ions moderately accelerate the spontaneous reaction, but do not effect a greater conversion. Manganese causes a very marked inhibition, while phenol, salicylaldehyde, and hemin completely prevent the reaction. Whenever inhibition occurs, the formation of both the ketone and the diols is retarded, or entirely suppressed.

A reaction mechanism involving the intermediary formation of a cholesterol 7-peroxide is proposed and discussed.

We wish to express our gratitude to Dr. N. H. Coy of the Biological Laboratories of E. R. Squibb and Sons for her invaluable cooperation in carrying out the numerous spectrographic measurements. We also acknowledge with thanks the able technical assistance of Miss Mildred Moore.

BIBLIOGRAPHY

1. Wintersteiner, O., and Bergström, S., *J. Biol. Chem.*, **137**, 785 (1941).
2. Bergstrom, S., and Wintersteiner, O., *J. Biol. Chem.*, **141**, 597 (1941).
3. Bergström, S., and Wintersteiner, O., *J. Biol. Chem.*, **143**, 503 (1942).
4. Blix, G., and Löwenhielm, G., *Biochem. J.*, **22**, 1313 (1928).
5. Wintersteiner, O., and Ritzmann, J. R., *J. Biol. Chem.*, **136**, 697 (1940).
6. Wintersteiner, O., and Ruigh, W. L., *J. Am. Chem. Soc.*, **64**, 1177 (1942).
7. Blumann, A., and Zeitschel, O., *Ber. chem. Ges.*, **46**, 1178 (1913).
8. Wienhaus, H., and Schumm, P., *Ann. Chem.*, **439**, 20 (1924).
9. Blumann, A., and Zeitschel, O., *Ber. chem. Ges.*, **47**, 2623 (1914).
10. Wallach, O., *Ann. Chem.*, **243**, 28 (1909).
11. Willstätter, R., and Sonnenfeld, E., *Ber. chem. Ges.*, **46**, 2953 (1913).
12. Kötz, A., and Richter, K., *J. prakt. Chem.*, **111**, 373 (1925).
13. Cook, A. H., *J. Chem. Soc.*, 1774 (1938).
14. Wienhaus, H., *Z. angew. Chem.*, **41**, 617 (1928).
15. Stephens, H. N., *J. Am. Chem. Soc.*, **50**, 568 (1928).
16. Hock, H., and Susemihl, W., *Ber. chem. Ges.*, **66**, 61 (1933).
17. McBain, J. W., in Kraemer, E. O., *Advances in colloid science*, New York, **1** (1942).
18. Criegee, R., Pilz, H., and Flygare, H., *Ber. chem. Ges.*, **72**, 1799 (1939).
19. Farmer, E. H., and Sundralingam, A., *J. Chem. Soc.*, 121 (1942).

AUTOXIDATION OF STEROLS IN COLLOIDAL AQUEOUS SOLUTION

IV. THE INFLUENCE OF ESTERIFICATION AND OF CONSTITUTIONAL FACTORS

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The ease with which cholesterol is converted into 7-ketocholesterol and 7-hydroxycholesterols by aeration in aqueous colloidal solution has stimulated a study of the behavior of cholesterol esters and of other unsaturated sterols in this reaction. The formation of 7-ketones and of the chromogenic diols was followed quantitatively as described in Paper III of this series (1). When the substrate was a sterol with a double bond in a position other than 5-6, at least the spectrographic method could be relied upon to give some indication whether oxidative attack had occurred, since α,β -unsaturated ketones are almost invariably formed in this type of autoxidation (see the "Discussion" in Paper III).

The cholesterol esters used were the acetate, palmitate, and olcate; the acid succinate was found to be unsuitable for our purpose, as it was partially hydrolyzed under the experimental conditions. The acetate and palmitate reacted with oxygen to form the ketone and chromogen, but at a very much slower rate than free cholesterol. The olcate yielded only traces of chromogen; the ketone could not be accurately determined in the autoxidized material from the olcate, because end-absorption obscured the characteristic maximum, but to judge from the extinction at $240\text{ m}\mu$ the amounts present were likewise extremely small. It is not quite clear to what extent the positive results were due to a gradual hydrolysis of the esters and concurrent oxidation of the free cholesterol thus formed. But whether or not the esters had reacted as such, esterification unquestionably provides strong protection against the oxidative attack. As already pointed out in Paper III, this holds true only for autoxidation in aqueous colloidal solution; by contrast, in organic solvents free cholesterol is resistant to oxygen, while the acetate was found to be readily oxidized (2).

The sterols investigated were stigmasterol, campesterol, fucosterol, 3(β)-hydroxy- Δ^5 -cholenic acid methyl ester, α -spinasterol, allocholesterol,

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and Δ^5 -cholestenediol-3,4(*cis*). Among these, the first four, which structurally correspond to cholesterol in that they possess a 5,6 double bond, were oxidized in the same manner as cholesterol; that is, with the formation of the 7-ketone and of chromogens. In the case of stigmasterol and campesterol (Δ^5 -24-epi-ergosterol) (3) the ketones were actually isolated. The reaction in all these instances took the typical course inasmuch as the formation of the autoxidation products came to a standstill after a few hours, as shown in Fig. 1 for stigmasterol. The final levels of Ke and Ch were generally lower than in the cholesterol experiments.¹ Though the plant sterols and the hydroxyeholenic acid ester did not emulsify as readily as cholesterol, insufficient dispersion does not seem to be the cause of the low yields. This follows from the experiment with stigmasterol, in which the products resulting from the autoxidation of stigmasterol were reemulsified and subjected to a second aeration. The levels of ketone and chromogen remained unchanged, indicating that, as in the case of cholesterol, the reaction is limited by the accumulation of the end-products.

The autoxidation products of α -spinasterol ($\Delta^{8:14,22:23}$ -stigmastadienol-3) (4) did not give the typical color in the Lifschütz reaction. However, the absorption measurements indicated that at least two α,β -unsaturated ketones, with maxima at 245 and 253 $m\mu$ respectively, had been formed. The isolation and characterization of these products had to be deferred for lack of sufficient starting material. It is planned to extend these studies to other similarly constituted sterols.

Allocholesterol showed no signs of being autoxidized; at least no light-absorbing substance was produced. The formation of chromogen could not be followed, as this compound itself gives (an atypical) color with Lifschütz's reagent. It therefore seems that the hydrogen atoms in position 6 are not sufficiently labilized by the proximity of the 4,5 double bond to permit attack by molecular oxygen.

The experiment with Δ^5 -cholestenediol-3,4 brought out the remarkable fact that the insertion of a hydroxyl group in position 4 of the cholesterol molecule deprives the latter of its reactivity. Δ^5 -Cholestenediol-3,4 did not yield a trace of 7-ketone on aeration, and the intensity of the Lifschütz reaction given by this sterol itself remained unchanged throughout the reaction period. It is possible that the glycolic arrangement of the two hydroxyl groups prevents the proper orientation of the molecule on the surface of the micelle, a factor which might also account for the behavior of the esters.

As far as the scope of the present study permits generalization, it may be said that the formation of substances giving the Lifschütz reaction is confined to sterols of the cholesterol type. The autoxidation reaction may

Ketone and chromogen are denoted by Ke and Ch, respectively, in this paper.

therefore serve as a useful implement in structural studies on sterols containing a cyclic double bond, the position of which is not known. The formation of Lifschütz-positive products may be considered indicative of the presence of a 5,6 double bond. Thus the present results confirm in this respect the structures assigned to fucosterol (5) and campesterol (3). The position of the second double bond present in fucosterol is not known, but work in progress in this laboratory indicates that it is not, as was originally supposed (6), located in the ring system, but in the side chain.

The formation of α,β -unsaturated ketones, as revealed by the spectrographic method, cannot similarly be used as a structural criterion. The result with α -spinasterol shows that a cyclic double bond in other, even in the so called inactive, positions may activate adjoining methylene groups sufficiently to make them susceptible to attack by oxygen. In this respect the autoxidation reaction is entirely equivalent to the chromic acid method commonly used for the introduction of a keto group in α position to a double bond. However, in some such cases it might be possible to derive information regarding the location of the double bond from the position of the maximum (7).

EXPERIMENTAL

Cholesterol Esters—The esters investigated were the acetate, palmitate, and oleate of cholesterol. Since the pH had to be kept near the neutral point to minimize saponification, Duponol PC, which gives more stable sols in this range than sodium stearate, was used as the detergent. In order to determine the extent of ester hydrolysis under the conditions of the autoxidation experiment, colloidal solutions of the acetate and palmitate were prepared with Duponol and kept at 85° under nitrogen for 5 hours. The detergent was removed in the usual way and the recovered material tested with digitonin for the presence of free cholesterol. The solution containing the acetate remained clear, and that of the palmitate merely deposited some crystals of the ester itself. A third ester which was tested in this fashion was the sodium salt of cholesterol acid succinate. In this case digitonin immediately gave a precipitate, indicating that extensive hydrolysis had occurred. The palmitate and oleate were free of chromogen; the acetate contained 2 per cent. The solutions, prepared by emulsifying 500 mg. of the esters with 1 gm. of Duponol in 1 liter of water, were aerated for 6 to 8 hours at 85°. A few figures will suffice to show that the autoxidation of the esters proceeds very much more slowly than that of free cholesterol.

Acetate, pH 7.4—After 4 hours the values for Ke and Ch were 4.8 and 11.5 per cent respectively. After 6 hours Ke had risen to 9 per cent. The preponderance of chromogen over ketone was noticeable also at the earlier time intervals.

Palmitate, pH 7.1—3 hours, Ke 4.1, Ch¹ 2.0 per cent; 5 hours, Ke 7.8, Ch 2.5 per cent; 8 hours, Ke 17, Ch 4.4 per cent.

Oleate, pH 7.5—5 hours (Ke 2.7), Ch 1.9 per cent; 8 hours (Ke 3.2), Ch 2.3 per cent. The ketone values as given are almost certainly too high, since the absorption maximum at 240 m μ was masked by end-absorption. The interfering substance was not present in the starting material.

The results are difficult to interpret in so far as, notwithstanding the control experiments with digitonin, it is conceivable that some ester hydrolysis may have taken place. In this case the autoxidation rate would be determined by that of the hydrolysis, and this seems to be borne out by the approximately linear character of the reaction curves (not counting the disproportional rise between the 5th and 8th hour in the experiment with the palmitate). Application of the digitonin reaction to the autoxidized samples was not attempted, since the digitonides of the ketone and the diols are much more soluble than that of cholesterol. At any rate the results leave no doubt that esterification of the hydroxyl group greatly restricts the ease with which the sterol in the colloidal state is attacked by oxygen.

Other Sterols—The sols were prepared with sodium stearate, as described for cholesterol in Paper III (1). It should be mentioned that none of the sterols emulsified as completely under these conditions as cholesterol. The solutions were more or less milky in appearance, while the cholesterol sols are so transparent that it is possible to use them directly for ultra-violet absorption measurements. In some instances there was a definite tendency towards flocculation or crystallization; this could be sometimes, but not always, prevented by adding more than the usual amount of alcohol.

All experiments were carried out at 85°. The values for ketone and chromogen formed are given as per cents of the starting material in order to make the results comparable with those obtained with cholesterol. Only compounds of the cholesterol type (5,6 double bond, 3-hydroxyl group) were found to give the typical color reaction with Lifschütz's reagent after aeration. The use of the standardization curve of 7(β)-hydroxycholesterol for computing the Ch content of the autoxidized material in these cases is justifiable on the basis of the colorimetric results obtained with other 7-hydroxysterols (Paper III, Table I). The only correction applied in calculating Ch was for the differences in molecular weight. The molecular extinction coefficient of 7-ketcholesterol used for computing the Ke content was assumed to be identical for all 7-ketosterols.

Stigmasterol (Fig. 1).—The curves showed no essential difference from

those obtained under identical conditions with cholesterol, except that the final levels of Ke and Ch were only about half as high.

The autoxidized material from both runs was recovered by ether extraction, combined, and after reemulsification again subjected to aeration at 85°. We hoped by this measure to raise the yields of autoxidation products, but as in the case of cholesterol (Paper III, Experiment 8) no further reaction took place. Ke and Ch remained stationary at the initial levels, which correspond approximately to those attained in the second run, since the bulk of the combined material was derived from the latter.

The sterol mixture was again recovered and separated with Girard's Reagent T into ketonic and non-ketonic fractions. The former on crys-

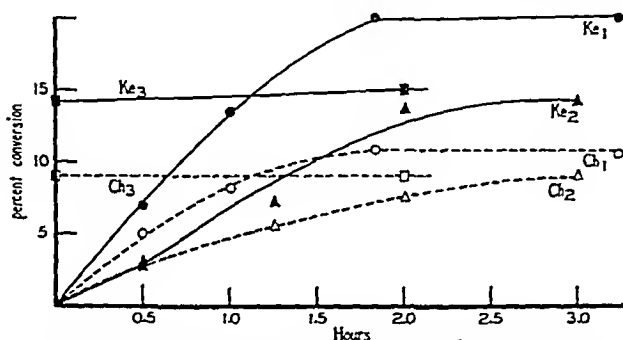


FIG. 1. Autoxidation of stigmasterol at 85°. Experiment 1, 500 mg. of stigmasterol in 70 cc. of alcohol; 20 cc. of sodium stearate in 1 liter of water; pH about 8.5; air. Experiment 2, 5 gm. of stigmasterol in 200 cc. of alcohol; 2 gm. of sodium stearate in 1 liter of water; pH about 8.5; air; emulsification poor, partial crystallization. Experiment 3, recovered material from Experiments 1 and 2 reemulsified with 300 cc. of alcohol, 4 gm. of sodium stearate, 1 liter of water. In spite of very slow addition there was partial crystallization. Aerated for 2 hours. Ke₁, Ke₂, Ke₃, ketone; Ch₁, Ch₂, Ch₃, chromogen in the respective experiments.

tallization from ether-pentane yielded platelets melting at 130–135°. Recrystallization did not improve the melting point, but the spectrum indicated that this product consisted essentially of the hitherto undescribed 7-ketostigmasterol (ϵ_{237} ; $\mu = 13,000$). It was converted with acetic anhydride and pyridine into the acetate, which after two recrystallizations from methanol melted at 184–186° (corrected). A mixture with an authentic preparation of 7-ketostigmasteryl acetate (m.p. 186.5°) showed no depression of the melting point.

$[\alpha]_D^{25} = -110^\circ$ (0.9% in chloroform); reference preparation, -109.3° ; $\epsilon_{237} = 12,300$

Analysis— $C_{27}H_{44}O_2$. Calculated. C 79.44, H 10.32
Found. " 79.12, " 10.09

The acetate was saponified with methanolic potassium carbonate solution (8). The free ketone was purified by precipitation with digitonin and repeated recrystallization, but the melting point remained unsharp as was the case with the original preparation (137°, after sintering at 120°).

Campesterol—1 gm. of campesterol (m.p. 158°), in 75 cc. of alcohol; 30 cc. of sodium stearate, 10 cc. of phosphate buffer in 1 liter of water; pH 9.0. The reaction took the usual course in that it became stationary after 3 hours, but the Ke to Ch ratio did not approximate 2 as in most other experiments at 85° (Ke 19, Ch 17 per cent).

The ketone was isolated in the form of the acetate as described in the experiment with stigmasterol. After three recrystallizations the melting point became constant at 165°. Later this compound, which had not been described before, was prepared by the chromic acid method (9). This preparation melted at 177–178° (corrected), showing that the keto acetate derived from the autoxidized sterol was still impure. This was confirmed by the low value of the molecular extinction coefficient (9000).

Fucosterol—400 mg. of fucosterol (m.p. 121°) in 75 cc. of alcohol; 20 cc. of sodium stearate in 1 liter of water; pH 8.2. The final levels, reached after 2½ hours, were 29 per cent for Ke and 14 per cent for Ch.

α-Spinasterol—200 mg. of α-spinasterol (m.p. 168°) in 50 cc. of alcohol; 70 cc. of sodium stearate, 10 cc. of phosphate buffer in 1 liter of water. The autoxidized material gave a brown instead of a blue pigment with Lifschütz's reagent. The spectrographic measurements revealed the presence of a substance absorbing light around 245 mμ; a control determination showed that the starting material was entirely transparent in the ultraviolet region. The intensity of this absorption became constant after 2 hours aeration with $\epsilon = 1300$, computed on the molecular weight of a ketospinasterol. The material was recovered and treated with Girard's reagent. The spectrum of the ketonic fraction (8.2 mg.) exhibited the original maximum at 245 mμ ($\epsilon = 3000$). However, most of the substance responsible for this absorption had remained in the non-ketonic fraction (158 mg., $\epsilon = 800$). Furthermore, a new peak at 253 mμ was clearly visible in the spectrum of this fraction. Further characterization of these products, obviously α,β-unsaturated ketones, will have to await the preparation of larger amounts of starting material.

3 (β)-*Hydroxy-Δ⁵-cholenic Acid Methyl Ester*—100 mg. of the ester in 10 cc. of alcohol; 3 cc. of sodium stearate, 1 cc. of phosphate buffer in 100 cc. of water; pH 8.1. A considerable portion of the ester remained unemulsified. The levels, reached after 2 hours, were 8 per cent for Ke and 10 per cent for Ch.

Allocholesterol—The spectrum remained blank. The chromogen value could be determined, as this compound itself gives a pink color with Lifschütz's reagent, which turns purple after a few minutes.

Δ^5 -Cholestenediol-3, 4(*cis*)—No light-absorbing substance was formed, and the chromogen value remained unchanged throughout the 5 hour reaction period on the level given by the starting material (29 per cent of that of 7(β)-hydroxycholesterol). The diol was recovered unchanged.

SUMMARY

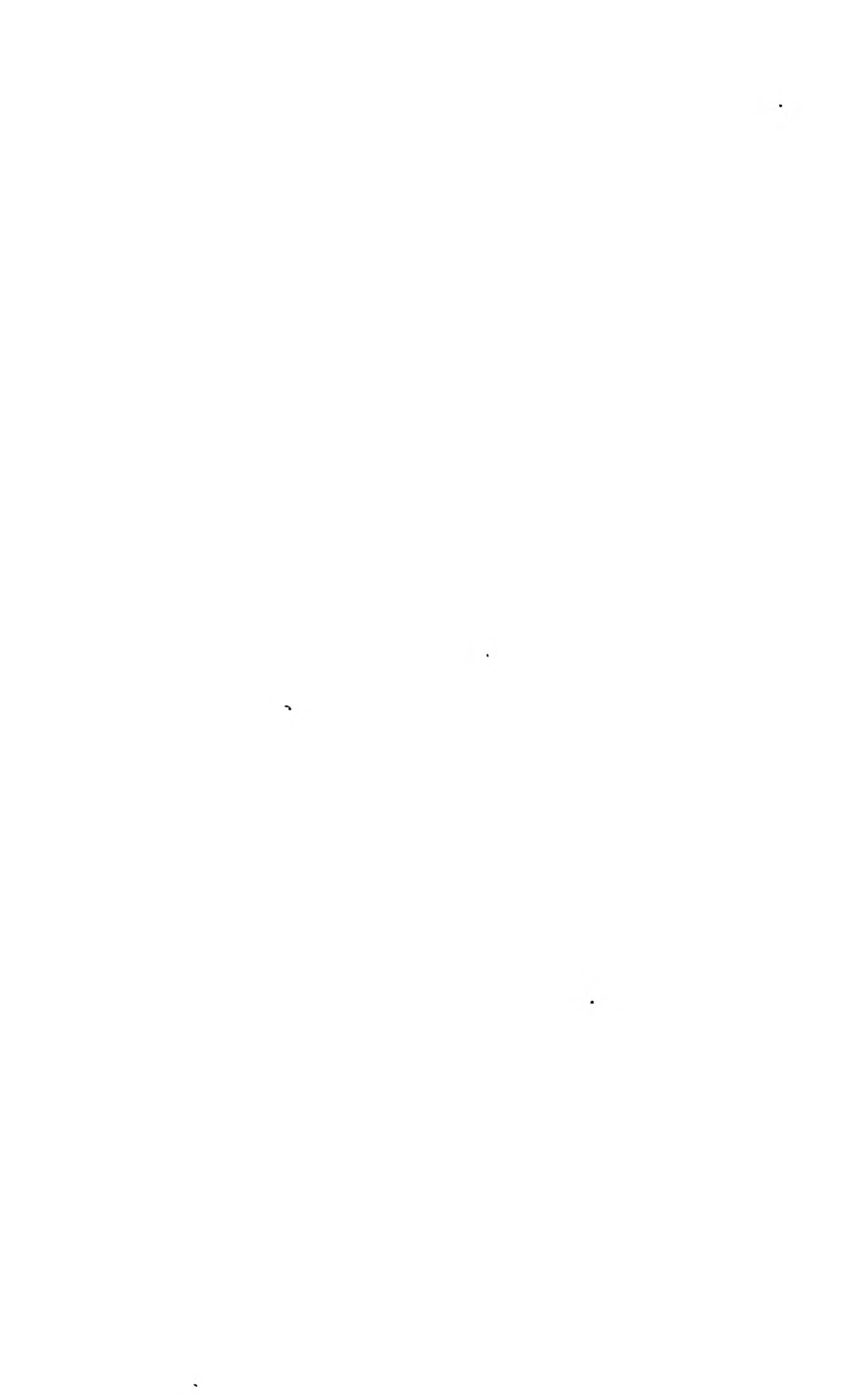
The course of the autoxidation of three esters of cholesterol in aqueous colloidal solution at 85° has been investigated. The results indicate that esterification greatly diminishes the susceptibility to attack by oxygen.

The behavior of several unsaturated sterols in this reaction has been studied. Compounds of the cholesterol type (stigmasterol, campesterol, fucosterol, 3(β)-hydroxy- Δ^5 -cholonic acid methyl ester) are oxidized in the typical manner to 7-ketones and chromogens. The reaction curves resemble those obtained with cholesterol, except that the final levels of ketone and chromogen were in all cases lower. Allocholesterol and Δ^5 -cholestenediol-3,4 gave no evidence of being autoxidized under these conditions. α -Spinasterol did not yield any Lifschütz-positive products, but the absorption spectra indicated that two different ketones, with maxima at 245 and 253 $m\mu$ respectively, had been formed.

We are greatly indebted to Dr. N. H. Coy of the Biological Laboratories of E. R. Squibb and Sons for the ultraviolet absorption measurements, and to Miss Mildred Moore for her able technical assistance. The analyses were carried out by Mr. J. F. Alicino, Fordham University.

BIBLIOGRAPHY

1. Bergström, S., and Wintersteiner, O., *J. Biol. Chem.*, **145**, 309 (1942).
2. Cook, A. H., *J. Chem. Soc.*, 1774 (1938).
3. Fernholz, E., and Ruigh, W. L., *J. Am. Chem. Soc.*, **63**, 1157 (1941).
4. Fernholz, E., and Ruigh, W. L., *J. Am. Chem. Soc.*, **62**, 2341 (1940).
5. Coffey, D. H., Heilbron, I. M., and Spring, F. S., *J. Chem. Soc.*, 738 (1936).
6. Coffey, D. H., Heilbron, I. M., Spring, F. S., and Wright, H. R., *J. Chem. Soc.*, 1205 (1935).
7. Woodward, R. B., *J. Am. Chem. Soc.*, **63**, 1123 (1941); **64**, 76 (1942).
8. Bergström, S., and Wintersteiner, O., *J. Biol. Chem.*, **141**, 597 (1941).
9. Ruigh, W. L., *J. Am. Chem. Soc.*, **64**, 1900 (1942).



THE INFLUENCE OF ENZYMES UPON THE ACTIVITY OF GONADOTROPIN OF PREGNANT MARE SERUM

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(Received for publication, July 8, 1942)

The references to enzymatic studies on gonadotropin of pregnant mare serum have been very few and the results recorded have only been of a qualitative nature. Cartland and Nelson (1) have reported that *emulsin* and *invertin* appear to be ineffective against gonadotropin of pregnant mare serum, whereas *trypsin* and *pepsin* inactivate it in 6 hours. Cartland and Nelson believed that the pepsin inactivation was possibly due to the acidity of the solution. Goss and Cole (2) and Evans, Gustus, and Simpson (3) have likewise reported on the trypsin inactivation. McShan and Meyer (4) have reported that preliminary experiments show that ptyalin destroys the activity of the hormone of pregnant mare serum but give no experimental details or results.

This study of the action of enzymes upon gonadotropin of pregnant mare serum was originally undertaken as a means of showing whether or not the carbohydrate component of the gonadotropin is essential for the activity of the hormone. The experiments were extended to study the effect of proteolytic enzymes in an attempt to note the presence or absence of certain groups or linkages in the protein moiety.

EXPERIMENTAL

Two preparations of gonadotropin of pregnant mare serum, one assaying 800 and the other 1800 i.u. per mg., were used in this study.

The ptyalin used in this experiment was prepared by acetone precipitation of pooled specimens of saliva. The acetone precipitate was extracted with H₂O and the aqueous solution stored in a frozen state. The preparation was active in digesting starch. 1 cc. of the ptyalin solution was added to 20 mg. of the hormone (800 i.u. per mg.) in 19 cc. of phosphate buffer at pH 8.8.

Emulsin was prepared as a crude enzyme from almonds. 2.0 mg. of the enzyme powder were added to 0.67 mg. of the hormone (1800 i.u. per mg.) dissolved in 10.0 cc. of NaOH-phthalate buffer at pH 5.2.

1.0 mg. of taka-diastase (Parke, Davis and Company) and 9.2 mg. of the hormone (1800 i.u. per mg.) were dissolved in 33.2 cc. of phosphate buffer at pH 6.62.

1.0 mg. of crystalline chymotrypsin and 4.1 mg. of the hormone (1800 i.u. per mg.) were dissolved in 14.35 cc. of phosphate buffer at pH 8.82.

1.0 mg. of commercial papain and 4.6 mg. of the hormone (1800 I.U. per mg.) were dissolved in 16.1 cc. of a citrate-phosphate buffer at pH 5.25.

1.0 mg. of crystalline pepsin and 4.3 mg. of the hormone (1800 I.U. per mg.) were dissolved in a citrate-phosphate buffer at pH 3.63.

1.0 mg. of trypsin (Wilson, 1:300) and 10 mg. of the hormone (800 I.U. per mg.) were dissolved in 10.0 cc. of phosphate buffer at pH 8.8.

1 cc. of an aqueous suspension of carboxypeptidase¹ and 11.8 mg. of the hormone (1800 I.U. per mg.) were added to 40.3 cc. of a phosphate buffer at pH 8.82.

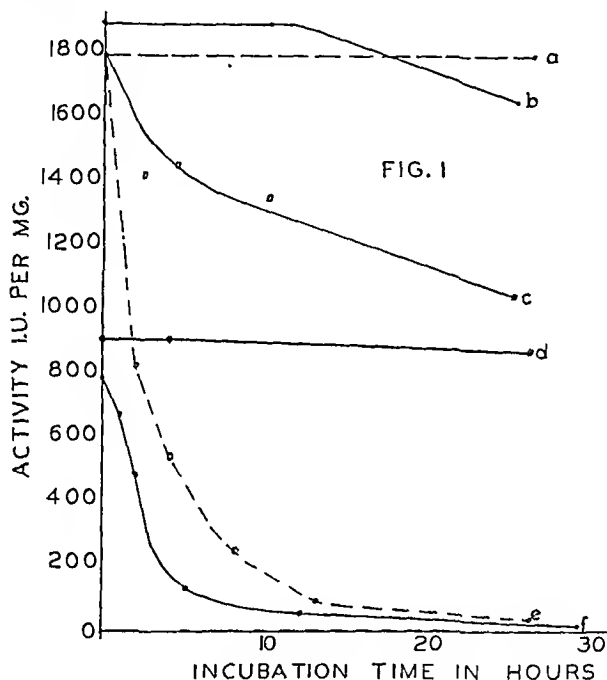


FIG. 1. Effect of amylolytic enzymes on gonadotropin of pregnant mare serum. Curve *a*, hormone plus phosphate buffer, pH 6.62; Curve *b*, hormone plus NaOH-phthalate buffer, pH 5.2; Curve *c*, hormone plus emulsin plus NaOH-phthalate buffer, pH 5.2; Curve *d*, hormone plus phosphate buffer, pH 8.8; Curve *e*, hormone plus taka-diastase plus phosphate buffer, pH 6.62; Curve *f*, hormone plus ptyalin plus phosphate buffer, pH 8.8.

The enzyme was added in each case to the hormone in the appropriate buffer solution and incubated at 38°. At varying time intervals, samples were withdrawn from the solution, the appropriate dilution made, and the solution frozen until the start of the assay. Assays were conducted by our routine procedure (5), with rats of the Sherman strain obtained from Rockland Farms. 400 rats were used in the experiments. The results of the experiments are shown in Figs. 1, 2, and 3.

¹ The enzyme suspension of carboxypeptidase was generously furnished to us by Dr. M. L. Anson of the Rockefeller Institute at Princeton.

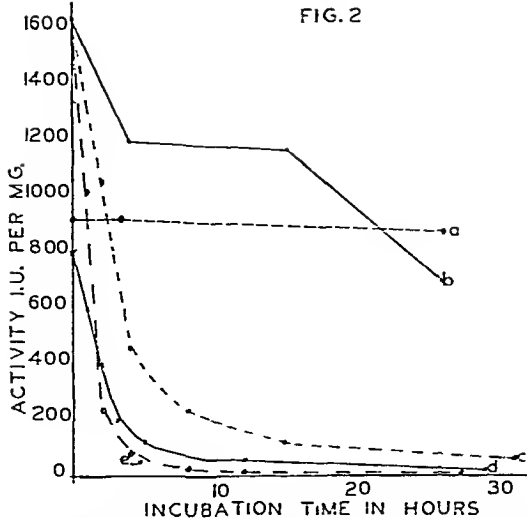


FIG. 2. Effect of proteolytic enzymes on gonadotropin of pregnant mare serum. Curve *a*, hormone plus phosphate buffer, pH 8.8; Curve *b*, hormone plus citrate-phosphate buffer, pH 5.25; Curve *c*, hormone plus papain plus citrate-phosphate buffer, pH 5.25; Curve *d*, hormone plus trypsin plus phosphate buffer, pH 8.8; Curve *e*, hormone plus chymotrypsin plus phosphate buffer, pH 8.8.

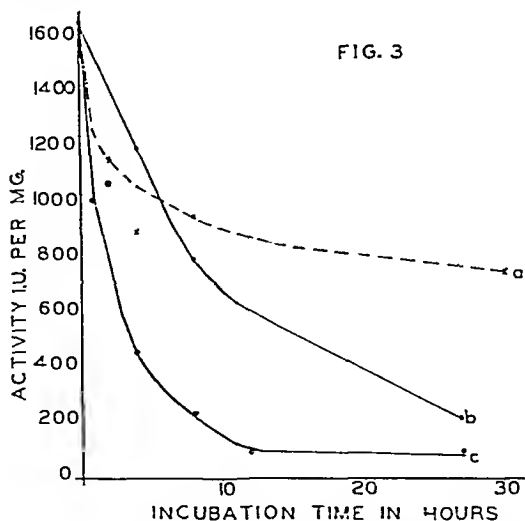


FIG. 3. Effect of proteolytic enzymes on gonadotropin of pregnant mare serum. Curve *a*, hormone plus carboxypeptidase plus phosphate buffer, pH 8.8; Curve *b*, hormone plus citrate-phosphate buffer, pH 3.63; Curve *c*, hormone plus pepsin plus citrate-phosphate buffer, pH 3.63.

DISCUSSION

It is evident from Fig. 1 that the amylolytic enzymes, ptyalin and taka-diastase, very rapidly inactivate the hormone. The enzyme emulsin inactivated about 30 per cent of the hormone in a period of 25 hours. Emulsin does not produce as rapid an initial inactivation as do ptyalin and taka-diastase.

It is evident that the carbohydrate portion of the hormone is one of the parts of the molecule that is essential for the activity of the hormone. The initial rapid inactivation by ptyalin suggests that the carbohydrate possesses α linkages; however, the slow inactivation by emulsin does not eliminate the possibility that there may be certain β linkages present.

Pepsin, whose optimum pH is in the acid range, when added to the hormone solution destroyed the gonadotropic activity at a faster rate than can be accounted for by the acidity of the buffer. This experiment indicates that pepsin itself will inactivate the hormone.

The present work (Figs. 2 and 3) indicates that all the proteolytic enzymes tested so far inactivate the hormone to about the same degree. Enzyme studies therefore indicate that the activity of pregnant mare serum can be considered to be a function of the whole molecule and not to be limited to either the protein or carbohydrate moieties.

The observation that the hormone of pregnant mare serum when dissolved in a citrate-phosphate buffer was inactivated at a faster rate than other buffer controls at about the same pH is of interest. Bowman (6) has pointed out that the activity of chorionic gonadotropin (prolan) decreased upon oxidation and that the activity of the preparation depended upon the oxidation-reduction state of the phenolic hydroxyl group of the tyrosine in the hormone molecule. It is possible that a similar situation is present in the hormone of pregnant mare serum, because unpublished data from this laboratory indicate that the treatment of the hormone with iodine leads to inactivation.

The work of MeShan and Meyer (4), showing that tryptic digestion of pituitary extracts leads to the preferential destruction of the luteinizing hormone, suggests that similar results might be expected with pregnant mare serum. At the present time we have been unable to show any selective destruction of either the follicle-stimulating or luteinizing factors in pregnant mare serum by our method of assay.

The susceptibility of a substrate to a given enzyme appears to be determined in the case of proteolytic enzymes by the presence of certain amino acid residues in the substrate, and in the case of amylolytic enzymes to depend upon the sugar residue and the type of linkages involved. Typical substrates (7) for pepsin and chymotrypsin have been shown to contain tyrosine or phenylalanine. However, pepsin has been shown to act on the

peptide linkages that involve the amino linkage of the typical amino acid, whereas chymotrypsin acts upon the peptide linkage that involves the carboxyl group of the typical amino acid. Trypsin acts at the carboxyl end of lysine or arginine residues.

Our results have indicated that all the enzymes tested have inactivated the hormone to a certain degree. The presence of tyrosine in the hormone of pregnant mare serum has already been pointed out (8). Lysine and arginine have been determined in this laboratory by the nitrogen distribution method as modified by Cavett (9), and the hormone assaying 800 i. u. per mg. was found to contain about 8.8 per cent lysine and 2.1 per cent arginine. The histidine content was 3.25 per cent.

SUMMARY

1. Ptyalin and taka-diastase rapidly inactivate the hormone of pregnant mare serum.

2. Emulsin will inactivate about 30 per cent of the hormone in a period of 25 hours at 38°.

3. Pepsin, chymotrypsin, trypsin, carboxypeptidase, and papain inactivate the hormone.

4. The activity of the hormone of pregnant mare serum is a function of the whole molecule, since the action of the enzymes on either the carbohydrate or protein portion of the molecule leads to inactivation.

5. The destructive influence of citrate buffers was noted.

6. A fraction of the hormone of pregnant mare serum assaying 800 i. u. per mg. was found to contain 8.8 per cent lysine, 2.1 per cent arginine, and 3.25 per cent histidine by a modified Van Slyke nitrogen distribution method.

BIBLIOGRAPHY

1. Cartland, G. F., and Nelson, J. W., *J. Biol. Chem.*, **119**, 59 (1937).
2. Goss, H., and Cole, H. H., *Endocrinology*, **15**, 214 (1931).
3. Evans, H. M., Gustus, E. L., and Simpson, M. E., *J. Exp. Med.*, **58**, 569 (1933).
4. McShan, W. H., and Meyer, R. K., *J. Biol. Chem.*, **126**, 361 (1938).
5. Cartland, G. F., and Nelson, J. W., *Am. J. Physiol.*, **122**, 201 (1938).
6. Bowman, D. E., *J. Biol. Chem.*, **137**, 293 (1941); **141**, 877 (1941).
7. Bergmann, M., and Fruton, J. S., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **1**, 63 (1941).
8. Li, C. H., Evans, H. M., and Wonder, D. H., *J. Gen. Physiol.*, **23**, 733 (1940).
9. Cavett, J. W., *J. Biol. Chem.*, **95**, 335 (1932).

LETTERS TO THE EDITORS

AN UNIDENTIFIED NUTRITIONAL FACTOR REQUIRED BY THE CHICK FOR FEATHER PIGMENTATION*

Sirs:

Failure to develop normally pigmented feathers has been observed at 6 weeks of age in Rhode Island Red chicks fed a basal diet containing 66.75 parts of degerminated yellow corn-meal, 15 of peanut meal, 10 of purified casein, 3 of soy bean oil, 0.25 of reinforced cod liver oil, and 5 of a salt mixture.¹ To each 100 gm. of this diet 300 γ of thiamine, 500 γ of riboflavin, 500 γ of pyridoxine, 700 γ of *d*-calcium pantothenate, and 0.5 gm. of glycine were added.

Similar results were obtained when this diet was supplemented (either singly or in combination) with 0.2 per cent choline chloride or with 1 γ of biotin given intramuscularly on alternate days. All chicks receiving these supplements showed abnormal feather pigmentation and development. When the basal diet was supplemented with 5 per cent dried brewers' yeast, no abnormalities in feather pigmentation and development were observed. The results are summarized in the accompanying table.

Supplement to basal diet (20 chicks per group)	Average weight at 6 wks	Chicks with abnormal feather pigmen- tation	Feather development
	gm	per cent	
None	268 \pm 28.0	100	Poor
0.2% choline chloride	346 \pm 75.4	100	"
1.0 γ biotin	256 \pm 64.8	100	"
0.2% choline chloride + 1.0 γ biotin	399 \pm 87.8	100	"
5% dried brewers' yeast	562 \pm 78.4	0	Excellent

The abnormal pigmentation affected most of the feathers, but was particularly striking in the wing and tail feathers and those of the lateral tracts. The tips of the primary wing and tail feathers, the development of which began in the fore part of the experiment, were red. As feather growth continued, only black pigment was deposited in many of these feathers. Finally when the chicks were depleted of their body stores of the protective factor, or as the requirement increased due to increased feather growth, no pigment whatever was deposited in the newly formed

* This work was made possible by the establishment of a Fellowship at Cornell University by the Grange League Federation Exchange, Inc., of Ithaca.

¹ Schumacher, A. E., and Heuser, G. F., *Poultry Sc.*, 19, 315 (1940).

part of the feather. Thus the same feathers often showed areas of red, black, and white. Many of the feathers which developed in the latter part of the experimental period were entirely white.

After the chicks were 6 weeks of age, a curative experiment of 4 weeks duration was conducted. Sixteen chicks that had the greatest amount of white feathers were selected and divided equally into two groups. One group was fed the basal diet plus 0.2 per cent choline chloride, while the other was fed the basal diet plus 5 per cent dried brewers' yeast. The chicks fed the basal diet plus choline continued to develop more white feathers. The new feathers of the chicks fed the yeast-supplemented diet were dark red, whereas other older feathers became red in the proximal portions, while the distal portions, which developed before the chicks were placed on the yeast-supplemented diet, were white.

The effectiveness of yeast was not due to a deficiency of pantothenic acid, since the basal diet contained more than an adequate amount of this vitamin. Hence, this abnormality in feather pigmentation is distinct from that reported by Groody and Groody.² It was also not caused by a deficiency of biotin, as the administration of this vitamin intramuscularly had no influence upon pigmentation.

Experimental work is being conducted to determine whether the preventive factor is *p*-aminobenzoic acid or inositol.

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² Groody, T. C., and Groody, M. E., *Science*, 95, 655 (1942).

FORMATION OF LACTIC ACID AND PYRUVIC ACID IN BLOOD CONTAINING PLASMODIUM KNOWLESI*

Sirs:

The blood of *Macacus rhesus* monkeys infected with malaria parasites (*Plasmodium knowlesi*) shows unusual ability to produce pyruvic acid *in vitro*. Blood samples from animals with varying degrees and stages of infection were defibrinated or heparinized and incubated (37.4°) with gentle rocking in flasks which contained alkali wells for absorption of CO₂. The first samples were analyzed about 10 minutes after the blood was drawn. Lactic acid was determined by a modified Friedemann-Cotonio-Shaffer method.¹ Pyruvic acid was determined by two methods² which gave similar results.

Typical results of nine of ten experiments are illustrated in Fig. 1, although not all experiments showed the relationships between pyruvate and lactate evident here. The maximum concentrations of pyruvic acid reached in blood from the ten infected monkeys were 15, 17, 19, 21, 36, 38, 41, 48, 50, and 57 mg. per cent. A correlation between the degree and stage of infection and the rate and extent of pyruvate formation has been noted. One sample of blood, with 23 per cent of the red cells infected with ring and small ameboid forms of the parasites, showed an increase of only 8 mg. per cent pyruvic acid in 3 hours; whereas a sample having approximately the same number of infected red cells, but containing predominantly mature parasites, showed a rise of 38 mg. per cent in 20 minutes. One sample of blood, which contained very high initial lactate and pyruvate, behaved exceptionally in that both lactate and pyruvate decreased from the beginning. The pyruvic acid concentration of normal monkey blood undergoes insignificant changes under comparable conditions.

In many experiments it has been noted that after 2 or 3 hours of incubation the *Plasmodia* have undergone degenerative morphological changes. Also, as early as 1 to 2 hours after blood is drawn, the rate of O₂ consumption usually begins to decrease. These facts suggest accumulation in the blood of a toxic substance. When it was found that freshly drawn blood contains large amounts of pyruvic acid, it seemed possible that this substance might be the responsible agent. Accordingly, we have attempted

* The experiments reported in this paper were aided by a grant from the Tennessee Valley Authority to the Department of Preventive Medicine of the University of Tennessee.

¹ Wendel, W. B., *J. Biol. Chem.*, 102, 47 (1933).

² Wendel, W. B., *J. Biol. Chem.*, 94, 717 (1931-32). Lu, G. D. *Biochem. J.* 33, 249 (1939).

to prevent accumulation of pyruvic acid by the following means: (a) incubation with 2 to 5 per cent CO_2 in O_2 , (b) incubation with bakers' yeast, (c) anaerobic incubation, (d) incubation with glycerol (250 mg. per cent), (e) incubation with methylene blue (0.003 per cent). The use of methylene blue is based upon the known ability of this dye to catalyze reduction of methemoglobin.³

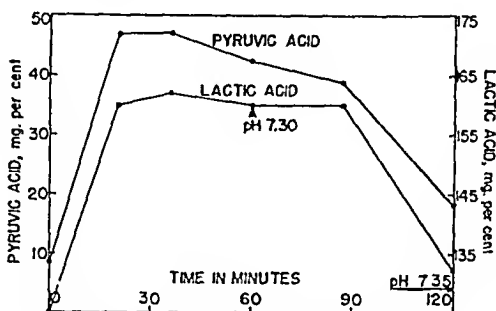


FIG. 1. Changes *in vitro* in concentration of lactic acid and pyruvic acid in blood containing *Plasmodium knowlesi*. Red cell count, 2,740,000; cells parasitized, 27 per cent.

Results—Carbon dioxide, yeast, and anaerobiosis, under the experimental conditions employed, did not prevent morphological changes, and of these agents only anaerobiosis prevented pyruvic acid formation *in vitro*. The sample of blood employed in the experiments with glycerol did not show usual morphological changes even in the control. Accordingly, the effect of glycerol upon morphology requires further study. It may be significant, however, that glycerol uniquely sustains respiration of this *Plasmodium*.

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Received for publication, August 3, 1942

³ For the literature see Wendel, W. B., *J. Clin. Inv.*, 18, 179 (1939).

SERINE INJURY*

Sirs:

In the course of experiments on the dietary factors affecting the composition of phospholipids in tissues, an injurious action of *dl*-serine on rats maintained on synthetic diets was observed. The data here presented suggest that the occurrence and gravity of the injury are strictly correlated with the nature of the diet and with the sex of the animal.

Two experimental diets were used. Diet 1¹ contained casein 10 parts, dextrin 37, sucrose 37, Crisco 5, cod liver oil 5, agar 2, salt mixture (Osborne and Mendel) 4. Diet 2 contained casein 30 parts, dextrin 27, sucrose 27, and otherwise was identical with Diet 1. Vitamin B complex was supplied in the diet in the form of dried yeast.

White rats weighing 90 to 100 gm. were placed on these experimental diets. After 7 days, various amounts of *dl*-serine² were administered daily by stomach tube as supplements to the experimental diets (see the table, Groups 1, 2, 3, 6, 7, 8). Other rats of the same weight were maintained on a stock diet³ with supplements of *dl*-serine (Groups 4, 5). The administration of serine was continued for a maximum period of 3 weeks when the animals survived. As controls for the technique of administration by stomach tube and for the possible effects of amino nitrogen supplied in the serine, several groups of rats (summarized as controls (a) in the table) received by stomach tube various other substances daily for 3 weeks. These include distilled water (3 cc.) and aqueous solutions of *dl*-alanine, ethanolamine, choline hydrochloride, and *dl*-methionine (50 to 150 mg.). Additional rats were maintained on Diet 1 and others on Diet 2 without supplementation to determine whether the experimental

* Aided by a grant from the Dazian Foundation for Medical Research. The valuable assistance of Mrs. Lillian Fishman is gratefully acknowledged.

¹ Most rats on Diet 1 grow for several weeks, though at a subnormal rate. Rats on Diet 2 exhibit normal growth, identical with that of rats on the stock diet.

² It seems very unlikely that the injurious effects observed were due to a contaminant in the serine. We used mainly a synthetic product manufactured by Merck and Company, Inc. Theoretical values were obtained on this material for total N, -COOH, and -NH₂ groups both before and after five successive recrystallizations, two from water and three from 50 per cent ethanol. Tests for traces of possible toxic substances in the original product were all negative. Furthermore, no qualitative or quantitative difference was observed in the action on rats by either Merck *dl*-serine, Eastman *dl*-serine, or Merck *dl* serine recrystallized five times.

³ Commercial diet of animal and vegetable origin containing 25 per cent protein, 5.5 per cent fat, 48 per cent carbohydrate, 11.25 per cent ash, according to Arcady Farms Milling Company, Chicago.

diets alone were injurious (summarized as controls (b)). Both control groups showed no visible ill effects.

The influence of diet and sex on the mortality is apparent from the table. In males, death usually resulted after 3 to 7 days of daily administration of serine in 100 mg. doses. All rats that received serine exhibited immediate cessation of growth and usually lost weight. However, those on the stock diet quickly resumed growth at a perfectly normal rate, though still receiving serine daily.

Mortality of Rats on Diets Supplemented with dl-Serine

Group No.	Sex	Diet No.	Serine daily	No of rats	No. of deaths
			mg.		
1	Male	1	50	7	1
2	"	1	100	10	8
3	"	2	100	13	12
4	"	Stock	50	5	0
5	"	"	100	7	0
6	Female	1	50	4	0
7	"	1	100	5	0
8	"	1	200	7	0
Controls (a)	Male	1		19	0
" (b)	"	1 and 2		12	0

Injury due to serine administration was characterized by anorexia, albuminuria, redness of the feet, hemorrhages under the nails. Autopsy findings on rats that died after administration of the amino acid pointed to a peripheral circulatory failure with marked congestion of the liver and lungs and severe damage to the renal tubules.

Further investigations on the mechanism of the injurious action of serine are now in progress.

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Received for publication, August 3, 1942

THE PREPARATION OF APPARENTLY NON-ANTIGENIC BEEF SERUM PROTEIN BY TREATMENT WITH ALKALI

Sirs:

In a recent communication, Davis and Eaton¹ have reported the preparation of beef serum albumin, beef serum, horse serum albumin, and horse serum of low antigenic power by treatment with alkali. We have been investigating the antigenicity of alkali-treated beef serum protein for the past several months, and the results thus far obtained confirm the observations of the above authors.

Preparation of Alkali-Treated Beef Serum Protein—The method used was essentially that employed by Johnson and Wormald² for the production of non-antigenic horse serum protein. Dried bovine serum was dissolved in 18 parts of 1 N sodium hydroxide solution. After the mixture had stood at room temperature for 27 hours, the pH was adjusted to 4.3 with hydrochloric acid, and the precipitated protein was removed by centrifugation. This material, after two washings with distilled water and drying with acetone, was used in the following experiments.

Failure to Produce Anaphylactic Reactions—A solution containing 2 mg. of the alkali-treated protein per cc. (pH 7.5) was prepared by dissolving the material in dilute sodium hydroxide solution. 0.5 cc. of this solution was injected intraperitoneally into each of six guinea pigs. Intracardial injections of 1 cc. were given to two of the animals after 18 days, to another two of them after 30 days, and to the remaining two after 46 days. No anaphylactic phenomena were observed.

Control guinea pigs, treated in the same way with solutions of the original dried beef serum, developed typical anaphylactic reactions at 30 days and at 46 days.

Failure to Produce Precipitins—A 7 per cent solution of alkali-treated protein was prepared (pH 7.5) and sterilized by candle filtration. Increasing amounts of this solution (0.5, 1, 2, and 4 cc.) were injected into the ear veins of two rabbits at intervals of 3 days. 6 days after the last injection, the sera of the rabbits were examined for precipitins, a wide range of antigen dilutions being used (1:250 to 1:128,000). The presence of precipitins could not be demonstrated.

Rabbit serum containing precipitins for beef serum reacted strongly with the original dried beef serum, but gave no reaction with the alkali-treated protein.

¹ Davis, H. A., and Eaton, A. G., *Proc. Soc. Exp. Biol. and Med.*, 50, 246 (1942).

² Johnson, L. R., and Wormald, A., *Biochem. J.*, 26, 1202 (1932).

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THE SYNTHESIS OF α -AMINO ACIDS FROM SUBSTITUTED ACETOACETIC ESTERS*

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(Received for publication, June 26, 1942)

The successes of Redemann and Dunn (1) and of Harington and Randall (2) in the synthesis of α -amino acids by the reduction of oximino acids led to a more detailed investigation of the preparation of oximino acids (or their esters) and their hydrogenation.

The synthesis of the oximes of α -keto esters from α -halogen esters and sodium nitrite was first carried out by Lepercq (3, 4). While yields up to 65 per cent have been obtained by this method, it does require first the preparation of the halogenated acid and also the lapse of considerable time, 3 to 4 weeks or longer. Thus, the procedure was abandoned for one more encouraging.

The use of substituted acetoacetic esters as intermediates in the synthesis of these oximes of α -keto acids has been previously reported. This method was first used in 1878 by Meyer and Züblin (5) who prepared ethyl α -oximinopropionate from methylacetoacetic ester by reaction with potassium nitrite in an acid medium. The same compound was prepared in better yields by Dieckmann and Groeneveld (6) who used sodium ethoxide and ethyl nitrite. Bouveault and coworkers, in 1904, published a series of papers (7-11) on the use of nitrosyl sulfate with alkyl-substituted acetoacetic esters and reported the synthesis of eight aliphatic α -oximino esters. The method was extended later by Wislicenus and Grützner (12) and by Hall, Hynes, and Lapworth (13). Recently McIlwain and Richardson (14) prepared α -oximinoglutaric acid and α -oximino- δ -chloro- γ -valerolactone in this manner and in 1939 Godfrin (15) followed the procedure for obtaining α -oximino acids.

Bouveault and Locquin (10, 11) reduced these oximino acids with zinc and hydrochloric acid, with sodium amalgam, and with aluminum amalgam but obtained uniformly unsatisfactory results. Other methods of reduction, both chemical and catalytic procedures, have been attempted with

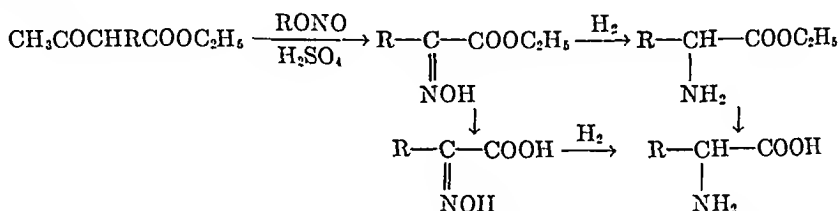
* Abstracted from a thesis submitted by Kenneth E. Hamlin, Jr., to the Faculty of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in June, 1941.

Presented before the Division of Biological Chemistry at the meeting of the American Chemical Society at Memphis, April, 1942.

† Wm. R. Warner Fellow, 1939-41.

varying degrees of success. However, Hartung (16) described a general method for the hydrogenation of oximes by means of palladium on charcoal; by employing ethanolic hydrogen chloride, the formation of secondary amines is prevented and excellent yields of primary amines were obtained.

It seemed likely that the reactions might be improved to eliminate certain objectionable features. Thus, it was found that the conveniently prepared and readily available butyl nitrite serves as an excellent nitrosating agent and that the α -oximino acids may be satisfactorily reduced and in good yield by the use of the palladium-charcoal catalyst in ethanolic hydrogen chloride. The complete reactions may be indicated in the accompanying formulas.



When the appropriately substituted acetoacetic esters are dissolved in 85 per cent sulfuric acid at 0° and treated with butyl nitrite, good yields of the corresponding α -oximino esters are obtained. These are readily hydrolyzed to the α -oximino acids. This procedure gives purer products and in equal or better yields (substantially higher for the aromatic derivatives) than the original of Bouveault. Table I lists the α -oximino acids and esters prepared in this manner.

Hydrogenation of the oximino acids or esters to the corresponding primary amino acids or esters was carried out by use of palladium on charcoal. Optimum conditions were reached with a hydrogen pressure of 10 atmospheres and with a concentration of hydrochloric acid in ethanol of 2 mole equivalents. Absorption of hydrogen at room temperature was quantitative in all cases and the resulting amino acids were isolated in good yields.

Ten α -amino acids have been prepared by this method (listed in Table II) and it seems probable that any monosubstituted acetoacetic ester may be converted into an α -amino acid in this way.

Individual products were examined in the field of a polarizing microscope in order to study their crystal habits. Photomicrographs are shown in Figs. 1 to 10.

EXPERIMENTAL

α -Bromo Esters—The general procedure of Zelinsky (25), with use of red phosphorus and bromine to convert organic acids to the α -bromo esters, was used.

TABLE I
 α -Oximino Acids (or Esters), $R-C(=NOH)-COOR'$

R	R'	Yield per cent	M p. (corrected) °C.	Recrystallized from	Crystal form from dioxane	Nitrogen	
						Calculated	Found
						per cent	per cent
CH ₃ —	—H	78	182, decomposition*	Ether-benzine	Monoclinic	10.69	10.91
"	—C ₂ H ₅	58	96†	Ligroin	Tetragonal	11.96	11.72
C ₂ H ₅ —	—H	80	155, decomposition†	Ether-benzine	Triclinic	10.89	10.15
n-C ₃ H ₇ —	"	85	145, " §	Ligroin	Monoclinic	9.65	9.50
n-C ₄ H ₉ —	"	80	137, "	Benzine	Tetragonal	9.65	9.50
sec-C ₄ H ₉ —	"	70	115, " ¶	Ligroin	Orthorhombic	6.15	6.15
C ₂ H ₅ OOCCH ₂ —	—C ₂ H ₅	85**	Oil	Benzine	Monoclinic	7.76	7.76
C ₂ H ₅ OOCCH ₂ CH ₂ —	"	91	82††	Ethanol-water	Orthorhombic	6.70	6.77
C ₆ H ₅ CH ₂ —	—H	80	168, decomposition‡‡	"	Monoclinic		
p-C ₆ H ₄ OC ₂ H ₄ CH ₂ —	"	87	157, " §§				

* Inglis and Knight (17) reported 180–181° with decomposition.

† Lepereq (3) gave 91.1° as the melting point

‡ Inglis and Knight (17) reported a melting point of 151°.

§ The melting point as given by Firth (18) is 143–144°.

|| Schmidt and Dieterle (19) reported 132° with decomposition.

¶ The melting point of Bouvenant and Locquin (10) as 161° was not duplicated after repeated recrystallization.

** Yield based on hydrogen absorbed during reduction to the amine.

†† The melting point checked that reported by Willems and Grätzner (12).

‡‡ Wolff (20) reported 167° with decomposition.

§§ Granochei *et al.* (21) recorded the melting point as 159°.

TABLE II
Amino Acids, $R-CH-COOH$
|
 NH_2

Amino acid	R	Yield <i>per cent</i>	Recrystallized from	Crystal form	Nitrogen		Derivative, m.p. (corrected)
					Calculated	Found	
Alanine	CH_3-	75	Water-ethanol	Tetragonal	15.72	15.52	Pierate of ethyl ester, 171°*
α -Aminobutyric	C_2H_5-	78	"	"	13.59	13.73	Benzoyl, 144°†
Norvaline	$n-C_3H_7-$	83	"	"	11.96	12.22	" 153.5°‡
Norleucine	$n-C_4H_9-$	85	"	"	10.68	10.73	<i>p</i> -Toluenesulfonamide, 122-124°†
Isoleucine	$sec-C_4H_9-$	80	"	"	10.68	10.91	" 138°†
Aspartic	$HOOCCH_2-$	69§	"	Triclinic	10.53	10.57	
Glutamic	$HOOCCH_2CH_2-$	74§	"	Tetragonal	9.52	9.70	
Phenylalanine	$C_6H_5CH_2-$	89	Water	Monoclinic	8.49	8.27	
O-Methyltyrosine	$p-CH_3OC_6H_4CH_2-$	85	"	Triclinic	7.18	7.15	

* Fischer (22) reported 171° as the melting point.

† The melting point checks that recorded by Shriner and Fuson (23).

‡ Slimmer (24) reported a melting point of 152.5°.

§ Represents combined yields of reduction and hydrolysis steps.



FIG. 1



FIG. 2

FIGS. 1 to 10. The amino acids were crystallized from water-ethanol in the field of a polarizing microscope. Magnification in all cases is 100 \times .

FIG. 1. Alanine; parallel extinction, tetragonal.

FIG. 2. α -Aminobutyric acid; parallel extinction, tetragonal.



FIG. 3



FIG. 4

FIG. 3. Norvaline; parallel extinction, tetragonal.

FIG. 4. Norleucine; parallel extinction, tetragonal.



FIG. 5



FIG. 6

FIG. 5. Isoleucine, parallel extinction, tetragonal.

FIG. 6. Aspartic acid; oblique extinction, birefringence, triclinic.

Acetoacetic Esters—In all cases the appropriate acetoacetic esters were synthesized according to the general method given in "Organic syntheses" (26), the suitable halide being added to a mixture of ethyl acetoacetate, sodium ethoxide, and absolute ethanol.

n-Butyl Nitrite—This was prepared by the method of Noyes (27). From 457 cc. of *n*-butyl alcohol were obtained 417 gm. of pure *n*-butyl nitrite, a yield of 81 per cent of the theory.

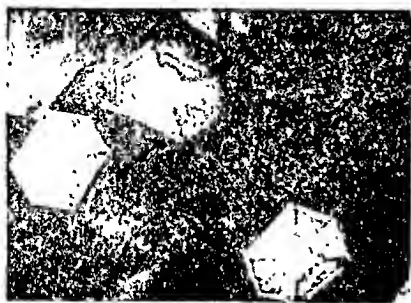


FIG. 7

FIG. 7. Glutamic acid; parallel extinction, tetragonal.



FIG. 8

FIG. 8. Phenylalanine; parallel and oblique extinction, birefringence, monoclinic.

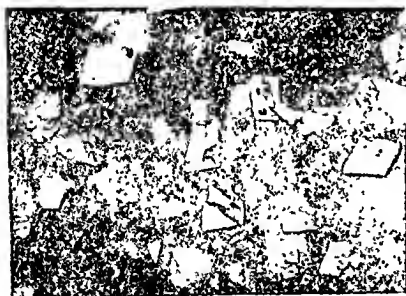


FIG. 9

FIG. 9. O-Methyltyrosine; oblique extinction, birefringence, triclinic.



FIG. 10

FIG. 10. Tyrosine; parallel extinction, tetragonal.

Ethyl α -Oximinopropionate (α -Bromo Ester Method)—The procedure described below is representative of the general method.

According to the method of Lepereq (3, 4), 100 gm. (0.55 mole) of ethyl α -bromopropionate were dissolved in 300 gm. of 95 per cent ethanol. To this solution was added a solution of 100 gm. of sodium nitrite in 300 cc. of water. The resulting mixture was allowed to stand at room temperature over a period of 30 days. At this time, the material was evaporated to

dryness *in vacuo* and the crystalline residue exhausted with ether. After the ether was distilled off, the product was recrystallized from ligroin. In this manner, yields between 50 and 65 per cent of ethyl α -oximinopropionate and ethyl α -oximinocaproate were obtained from the corresponding α -bromo esters.

α -Oximinopropionic Acid—The procedure used here served as a general method for the preparation of all oximino acids used and is described in detail.

30 gm. of 85 per cent sulfuric acid (about twice the weight of the ester used) were placed in a 400 cc. beaker, surrounded by an ice-salt bath. Mechanical stirring was provided and the temperature of the reaction mixture was maintained at -5° to 0° . Then, 14.4 gm. (0.1 mole) of ethyl methyl acetoacetate (b.p. 57.5° , 1 mm.) were added slowly, the temperature of the mixture being kept below 0° . After addition of the ester, 11 gm. (0.1 mole plus a 5 per cent excess) of butyl nitrite were added dropwise, the temperature being maintained below 0° . After addition of the nitrite, crushed ice was added to the acid mixture. At this point, a white, curdy mass of ethyl α -oximinopropionate precipitated. Since, in this individual case, the ester has a relatively high melting point, it was found more suitable to isolate the ester. However, generally, the mixture was next extracted with ether. The ethereal portion then was thoroughly extracted with 10 per cent sodium hydroxide. To obtain the free oximino acid, the alkaline extract was heated on a steam bath for 10 minutes. After it was cooled and carefully acidified with concentrated hydrochloric acid, a portion of the α -oximinopropionic acid precipitated. Because of the high solubility of this acid, it was necessary to salt-out the product and extract well with ether. The free oximino acid then was obtained by removing the ether and recrystallizing from a suitable solvent.

Palladium-Charcoal Catalyst—The palladium-charcoal catalyst was prepared after the manner of Hartung (16) and dried *in vacuo* over sulfuric acid before use.

Alanine—The procedure described below served as a general method for the hydrogenation of all oximino acids and is described in detail.

To 5.15 gm. (0.05 mole) of α -oximinopropionic acid were added 3 gm. of the palladium catalyst (1:10). An additional 0.5 gm. of palladium chloride was added and 100 cc. of 95 per cent ethanol. About 10 cc. of 35 per cent hydrochloric acid (0.11 mole) was added and the entire mixture was placed in a glass liner, fitted for use in a pressure hydrogenator bomb. The mixture was shaken at a pressure of 10 atmospheres of hydrogen, until reduction was complete. Hydrogenation to the half-way point was rapid, about 30 minutes being necessary. The second step went much more slowly, about 3 hours being required for the theoretical quantity of hydrogen to be

absorbed. After the catalyst was filtered off and washed with additional alcohol, the solvent was removed from the filtrate and washings *in vacuo*. The white crystalline residue was dissolved in a minimum of distilled water and filtered. The clear filtrate was heated to boiling and 28 per cent ammonium hydroxide was added to the isoelectric point. At this point, white crystals of alanine began to preecipitate. 3 volumes of 95 per cent ethanol were added to the hot solution and the mixture was chilled in the refrigerator for 12 hours. After the preecipitate was removed, a second crop of alanine was reecovered from the mother liquor.

When reduction of the α -oximino ester was carried out, the hydrochloride of the amino acid ester was obtained. Isolation of the free ester was carried out by the method of Adkins and McElvain (28).

Hydrolysis of the ester was accomplished by refluxing in distilled water until the alkaline reaction had disappeared. On concentration, crystalline alanine separates out.

Tyrosine—2 gm. of O-methyltyrosine were heated in a sealed tube with 25 cc. of concentrated hydrochloric acid at 180° for a period of 3 hours. The solvent was removed *in vacuo* from the demethylated product and the residue taken up in a minimum of water. The solution was brought to the isoelectric point and tyrosine was isolated in an 85 per cent yield (1.3 gm.).

$C_9H_{11}O_3N$. Calculated, N 7.73; found, N 7.43

SUMMARY

The synthesis of α -amino acids through the nitrosation of appropriately substituted acetoacetic esters followed by hydrolysis and hydrogenation is described. Alanine, α -aminobutyric acid, norvaline, norleucine, isoleucine, aspartic acid, glutamic acid, phenylalanine, and O-methyltyrosine were synthesized in good yield by this method. O-Methyltyrosine was demethylated and converted into tyrosine. It appears likely that any monosubstituted acetoacetic ester may be converted into an α -amino acid by an application of these reactions.

Photomicrographs of the amino acids synthesized were prepared, the compounds being crystallized in the field of a polarizing microscope.

BIBLIOGRAPHY

1. Redemann, C. E., and Dunn, M. S., *J. Biol. Chem.*, **130**, 341 (1939).
2. Harington, C. R., and Randall, S. S., *Biochem. J.*, **25**, 1917 (1931).
3. Lepereq, M. G., *Bull. Soc. chim.*, **9**, 630 (1893).
4. Lepereq, M. G., *Bull. Soc. chim.*, **11**, 297, 886 (1894).
5. Meyer, V., and Züblin, J., *Ber. chem. Ges.*, **11**, 693 (1878).
6. Dieckmann, W., and Groeneveld, A., *Ber. chem. Ges.*, **33**, 600 (1898).
7. Bouveault, L., and Wahl, A., *Bull. Soc. chim.*, **31**, 677 (1904).
8. Locquin, R., *Bull. Soc. chim.*, **31**, 1070 (1904).

9. Bouveault, L., and Loequin, R., *Bull. Soc. chim.*, **31**, 1153 (1904).
10. Bouveault, L., and Loequin, R., *Compt. rend. Acad.*, **141**, 116 (1905).
11. Bouveault, L., and Loequin, R., *Bull. Soc. chim.*, **35**, 965 (1906).
12. Wislicenus, W., and Grützner, R., *Ber. chem. Ges.*, **42**, 1940 (1909).
13. Hall, N., Hynes, J. E., and Lapworth, A., *J. Chem. Soc.*, **107**, 136 (1915).
14. McIlwain, H., and Richardson, G. M., *Biochem. J.*, **33**, 44 (1939).
15. Godfrin, M. A., *J. pharm. et chim.*, **30**, 321 (1939).
16. Hartung, W. H., *J. Am. Chem. Soc.*, **50**, 3370 (1928).
17. Inglis, J. K. H., and Knight, L. E., *J. Chem. Soc.*, **93**, 1595 (1908).
18. Fürth, A., *Ber. chem. Ges.*, **16**, 2180 (1883).
19. Schmidt, J., and Dieterle, H., *Ann. Chem.*, **377**, 59, 63 (1910).
20. Wolff, L., *Ann. Chem.*, **399**, 314 (1913).
21. Gränocher, C., Gerö, M., Ofner, A., Kloppenstein, A., and Schlatter, E., *Helv. chim. acta*, **6**, 458 (1923).
22. Fischer, E., *Ber. chem. Ges.*, **34**, 443 (1901).
23. Shriner, R. L., and Fuson, R. C., *The systematic identification of organic compounds*, 2nd edition, New York, 204, 205 (1940).
24. Slinnmer, M. D., *Ber. chem. Ges.*, **35**, 405 (1902).
25. Zelinsky, N., *Ber. chem. Ges.*, **20**, 2026 (1887).
26. Marvel, C. S., and Hager, F. D., in Whitmore, F. C., *et al.*, *Organic syntheses*, New York, **7**, 36 (1927).
27. Noyes, W. A., *J. Am. Chem. Soc.*, **55**, 3888 (1933).
28. Adkins, H., and McElvain, S. M., *Practice of organic chemistry*, New York, **229** (1925).

PHOTOMETRIC DETERMINATION OF ARGININE

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The intensity of color developed by arginine in the modification of the Sakaguchi reaction described by Weber (1) has been shown by Jorpes and Thorén (2) to decrease in linear fashion with increase in the amount of arginine employed for analysis. Extrapolation to zero concentration should, therefore, give a close approximation to the true arginine value (*cf.* Table I, Solution I).

We have found that the inhibition of color development by ammonia and by histidine, observed by Weber, likewise follows a linear course. The extent of this inhibition depends on the amounts of interfering substances but is independent of the arginine concentration.¹ Extrapolation to zero concentration always results in the same color value per unit weight of arginine, as shown in Table I, Solutions II to V. These observations should be applicable to protein hydrolysates.

In the determination of arginine in HCl hydrolysates of β -lactoglobulin² (3-5) we found decreasing percentages when increasing amounts of the hydrolysate were taken for analysis. When the apparent arginine content was plotted against the amount of protein used for analysis, the values fell on a straight line. Extrapolation to zero protein concentration should, in view of the experiments with arginine in the presence of NH_3 and histidine in Table I, give a reliable value for the arginine content. For β -lactoglobulin (Fig. 1) this procedure yields an arginine content of 2.87 per cent compared to 2.79 per cent obtained by isolation as the monoflavinate by Cannan *et al.* (4) and to 2.66 per cent as the diflavinate (6) by Vickery (*cf.* (4)). The agreement of the colorimetric and isolation procedures is satisfactory, especially as the diflavinate value, which includes no solubility correction, is certainly a minimum.

The results for a number of highly purified crystalline proteins are reported in Fig. 1, where the apparent arginine content is plotted against the corresponding amount of protein used for analysis. The individual points are the average of independent determinations obtained on two to

¹ Weber's data ((1) Table II), as far as they go, are in complete agreement with this finding.

² We are indebted to Dr. R. K. Cannan for a preparation of β -lactoglobulin and for information on the amide and basic amino acid content of β -lactoglobulin in advance of publication.

four separate hydrolysates. The values for the individual proteins fall on lines which are also straight but differ in slope, owing presumably to the influence of differing amounts of interfering substances. Horse serum albumin B (carbohydrate-free, *cf.* (8), for which we are indebted to Mr. Manfred Mayer of this Department) had an arginine content³ of 5.52 per cent, corresponding to 22 residues per mole (mol. wt. = 70,000). Omitted from Fig. 1 are the very similar data (arginine content³ 5.49 per cent) on horse serum albumin A, the carbohydrate-containing fraction, for which we are indebted to Dr. Hans Neurath. Human serum albumin (prepared by Dr. F. E. Kendall (9)) had a slightly higher arginine content,³ 6.30 per cent, corresponding to 25 residues per mole (mol. wt. = 70,000). The

TABLE I

Color Development with Different Amounts of Arginine, Alone and in Presence of NH₃ and Histidine; Total Volume 10 Cc.

Solution	K per 0.1 mg. arginine per cc. solution analyzed				Extrapolated to 0 cc. solution,* K ₀ per 0.1 mg. arginine
	1 cc.	1.5 cc.	2.5 cc.	3 cc.	
I. 0.020 mg. per cc. arginine	1.668	1.636	1.588	1.560	1.71 ₈
II. 0.022 " " " " +	1.63	1.58	1.52	1.47	1.71
0.011 " " " NH ₃ -N					
III. 0.022 " " " arginine +	1.59	1.53	1.43	1.36	1.70
0.011 " " " NH ₃ -N +					
0.049 " " " histidine					
IV. 0.022 " " " arginine +	1.59	1.49	1.40	1.33	1.70
0.022 " " " NH ₃ -N					
V. 0.022 " " " arginine +	1.57	1.45	1.31	1.25	1.71
0.155 " " " histidine					

* Calculated according to "least squares." Cc. of solution = X , K per 0.1 mg. observed = Y .

$$K_0 \text{ per 0.1 mg.} = \frac{18.50\sum Y_i - 8\sum X_i Y_i}{10}$$

crystalline enzymes were kindly placed at our disposal by Dr. M. Kunitz. Ribonuclease, trypsinogen,⁴ and trypsin⁴ contain 5.16, 1.61, and 3.27 per cent respectively of arginine.³

The arginine content³ of ehymotrypsinogen is 2.83 per cent, corresponding to 6 (5.97 calculated) residues of arginine per mole on the basis of a molecular weight of 36,700 (established by amino acid analysis (7) and osmotic pressure measurements (10, 11)). The close approximation to an

³ On a moisture-, ash-, and sulfate-free basis. The sulfate content of these proteins was established in a separate determination (*cf.* (7)) and will be reported in connection with other data.

⁴ The tryptophane content of trypsinogen is 2.9 per cent compared to 4.6 per cent for trypsin. Other preparations of these proteins will be investigated.

integral value is in this case additional evidence for the precision of the present method. Arginine determinations in chymotrypsinogen were also carried out in the presence of added arginine with satisfactory results (Table I).

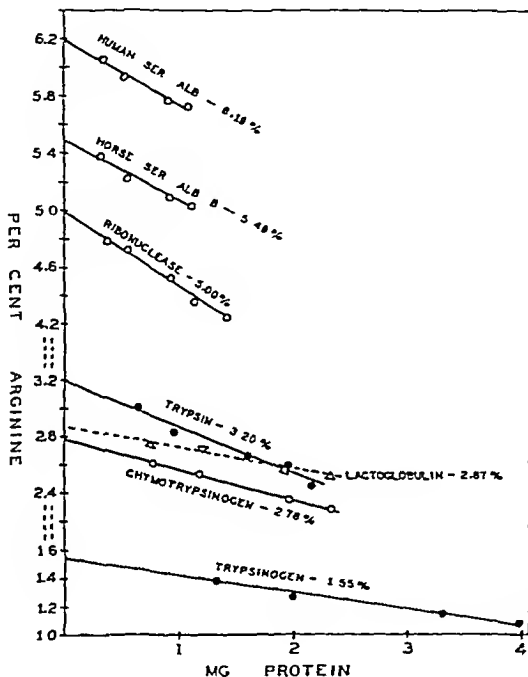


FIG. 1 Arginine content of various crystalline proteins. The values reported in the chart are not corrected for the sulfate content of these proteins. On a sulfate-free basis (*cf.* (7)) the arginine content is as follows: trypsinogen 1.61, chymotrypsinogen 2.83, β lactoglobulin 2.87, trypsin 3.27, ribonuclease 5.16, horse serum albumin B 5.52, human serum albumin 6.30 per cent.

Crystalline pepsin, for which we are indebted to Dr. R. M. Herriott, contains 2 residues of arginine per mole. A preparation of native swine pepsin, containing a small amount of non-protein nitrogen, had an arginine content³ of 1.01 per cent. The same preparation after denaturation and removal of the non-protein nitrogen showed an arginine content³ of 0.96 per cent (the data are omitted from Fig. 1).

The experiments reported in this paper indicate that the photometric

determination of arginine by the Sakaguchi reaction can be carried out in protein hydrolysates with considerable accuracy. The main advantage of the method is that it permits the determination of arginine with relatively small amounts (10 to 40 mg.) of protein.

EXPERIMENTAL

The procedure is essentially that of Weber (1), but certain modifications introduced by Jorpes and Thorén (2) and by Thomas, Ingalls, and Luck (12) are incorporated. The photometric determinations were carried out with a Zeiss step-photometer, with Filter S-50 (*cf.* (2)). The terms "extinction coefficient (K)" and " K per mg." are used as defined in a previous publication (13).

Solutions—(a) NaOH, a 10 per cent solution.

(b) α -Naphthol. The stock solution contains 0.25 per cent of purified α -naphthol in 95 per cent alcohol, and is kept cold in the dark. For use, the stock solution is diluted with water 1:4.

(c) Sodium hypobromite, 2.5 per cent (by weight) of bromine in 5 per cent NaOH. This solution is kept cold in the dark and its titer is checked iodometrically about once a week.

(d) Urea. A 40 per cent solution of purified urea in water.

Procedure—All solutions are held in an ice bath before the determinations are started. Place 5 cc. of neutralized protein hydrolysate (diluted as given under "Hydrolysis") in a graduated cylinder and cool for 30 to 60 minutes; add 1 cc. each of solutions (a) and (b), mix, and cool for 5 to 10 minutes. Then add with vigorous shaking the required amount (0.15 to 0.75 cc.) of solution (c), followed in exactly 15 seconds by 1 cc. of solution (d); shake, dilute immediately to 10 cc. with ice-cold water, and read the cold solution within about 1 to 3 minutes in the photometer. The determinations should be carried out in a relatively dry atmosphere. If the humidity is too high, moisture will deposit on the photometer cups.

Since maximal color development is obtained only with a definite amount of hypobromite, this has to be carefully determined for the successive amounts of hydrolysate used for the determinations. For example, 1, 1.5, 2.5, and 3 cc. of a hydrolysate of β -lactoglobulin (containing 0.767 mg. per cc.) required 0.25, 0.25, 0.35, and 0.40 cc. of hypobromite respectively for maximum color development.

Calibration—In agreement with Jorpes and Thorén (2) we find that the extinction coefficient K per 0.1 mg. of arginine increases with decreasing amounts of arginine and that the points fall on a straight line. Two preparations of arginine monohydrochloride of known purity were used in these determinations. The value of K in a total volume of 10 cc. was determined for four amounts of arginine: 0.02 ($K = 0.333_s$) and 0.03 mg. ($K = 0.490_s$)

in vessels of 1.000 cm. length, and 0.05 ($K = 0.794_3$) and 0.06 mg. ($K = 0.936_2$) in vessels of 0.500 cm. length. The average values of K per 0.1 mg. of arginine (Table I, Solution I) are slightly higher than those reported by Jorpes and Thorén (2) because we used 2.5 times as large an amount of α -naphthol (*cf.* (12)). When the standardization was carried out exactly according to Jorpes and Thorén, the same values were obtained as reported by these authors. Similar calibrations can be established for other instruments. Inhibition of color development by NH_3 and histidine is shown by the other data in Table I.

It can be seen that mathematical extrapolation to zero concentration gives the same color value per unit weight of arginine (K_0 per 0.1 mg. of arginine = 1.70 to 1.72) regardless of the presence of histidine and ammonia.

TABLE II

Recovery Experiments; Arginine Added to Hydrolysate of Chymotrypsinogen (Arginine Content 2.78 Per Cent)

Protein (1)	Color development* (2)	Arginine in protein (3)	Arginine added (4)	Arginine expected† (5)	Arginine found (6)	Recovery of added arginine (7)
mg.	per cent	γ	γ	γ	γ	per cent
0.575	95.5	16.0	15.0	29.6	29.7	101
0.960	92.5	26.7	25.0	47.8	48.0	101
1.15	91.0	32.9	32.0	56.4	56.5	100

* From a plot of the chymotrypsinogen data in Fig. 1.

$$\dagger (5) = \frac{((3) + (4)) \times (2)}{100}$$

Hydrolysis—From 10 to 40 mg. of finely powdered protein of known moisture content (*cf.* (7)) are hydrolyzed with 3 cc. of 6 N HCl and 1 drop of octyl alcohol for 16 hours in an oil bath at 130–140° in an inert atmosphere. The hydrolysate is almost neutralized with 6.8 cc. of 2.5 N NaOH with cooling, and diluted with water so that 1 cc. contains close to 0.02 mg. of arginine (on the basis of a preliminary determination). For example, 19.8 mg. of β -lactoglobulin (moisture content 7.01 per cent) were hydrolyzed with 3 cc. of HCl, neutralized, and diluted to 24 cc. The determinations are then carried out with 1, 1.5, 2.5, and 3 cc. of the diluted hydrolysate, the volume in each case being adjusted to the 5 cc. required in the procedure. The apparent arginine content is plotted against amount of protein analyzed, as shown in Fig. 1; extrapolation to zero (either graphically or by calculation) gives the true arginine content.

When solutions of arginine monohydrochloride were subjected to such hydrolysis, no measurable destruction was observed.

Recovery of Added Arginine—From 15 to 32 γ of arginine were added to varying amounts of a chymotrypsinogen hydrolysate. The color development (Table II, Column 2) corresponding to the particular amounts of chymotrypsinogen was ascertained from a plot of the data in Fig. 1.

SUMMARY

The intensity of the color developed by arginine in the Sakaguchi reaction decreases in a linear fashion with increasing amounts of arginine. The inhibition of color development by NH_3 and by histidine likewise follows a linear course. Extrapolation to zero concentration results in the same color value per unit weight of arginine.

In order to establish the arginine content of a protein, about four different amounts of a hydrolysate are analyzed and the apparent arginine content is plotted against the corresponding amount of protein used for analysis. Extrapolation to zero protein concentration either graphically or by calculation gives the true arginine content.

The following highly purified crystalline proteins were investigated; the arginine content on a dry, ash-free and sulfate-free basis is given in parentheses: swine pepsin (0.96 per cent, 2 residues per mole), trypsinogen (1.61 per cent), chymotrypsinogen (2.83 per cent, 6 residues per mole), β -lactoglobulin 2.87 per cent, 7 residues per mole), trypsin (3.27 per cent), ribonuclease (5.16 per cent), horse serum albumin A (5.49 per cent, 22 residues per mole), horse serum albumin B (5.52 per cent, 22 residues per mole), human serum albumin (6.30 per cent, 25 residues per mole).

BIBLIOGRAPHY

1. Weber, C. J., *J. Biol. Chem.*, **86**, 217 (1930).
2. Jorpes, E., and Thorén, J., *Biochem. J.*, **26**, 1504 (1932).
3. Palmer, A. H., *J. Biol. Chem.*, **104**, 359 (1934).
4. Cannan, R. K., Palmer, A. H., and Kibrick, A. C., *J. Biol. Chem.*, **142**, 803 (1942).
5. Warner, R. C., and Cannan, R. K., *J. Biol. Chem.*, **142**, 725 (1942).
6. Vickery, H. B., *J. Biol. Chem.*, **132**, 325 (1940).
7. Brand, E., and Kassell, B., *J. Gen. Physiol.*, **25**, 167 (1941).
8. Brand, E., and Kassell, B., *J. Biol. Chem.*, **141**, 999 (1941).
9. Kendall, F. E., *J. Biol. Chem.*, **138**, 97 (1941).
10. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1935).
11. Kunitz, M., *J. Gen. Physiol.*, **22**, 207 (1938).
12. Thomas, L. E., Ingalls, J. K., and Luck, J. M., *J. Biol. Chem.*, **129**, 263 (1939).
13. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115 (1938).

ANALYSIS AND MINIMUM MOLECULAR WEIGHT OF β -LACTOGLOBULIN

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In 1934 Palmer (1), in R. K. Cannan's laboratory, succeeded in obtaining from the plasma of cow's milk a crystalline protein with the general properties of a globulin, which was subsequently designated as β -lactoglobulin (2). According to Palmer (1) and to Sørensen and Sørensen (3) from 50 to 60 per cent of the whey proteins can be obtained in the form of this globulin. Detailed physicochemical investigations (solubility (1), electrophoretic mobility (4), ultracentrifuge studies (2, 4), dissociation tendency (5)) indicate the homogeneity of β -lactoglobulin by criteria at present available. Pedersen's ultracentrifuge investigations on skim milk (2) show that β -lactoglobulin is a protein native to milk and is not an artifact produced during isolation and crystallization.

This paper deals with the determination of cysteine, cystine, methionine, tyrosine, tryptophane, arginine, and threonine in β -lactoglobulin.¹ From these data a value for the minimum molecular weight ($M_{\min.}$) is obtained which is in close agreement with the molecular weight in solution computed from ultracentrifuge data (4). The number of amino acids per mole of β -lactoglobulin is ascertained with the aid of additional information¹ (amide N and basic amino acid content (6, 7)). The rôle of the hydroxyl groups is discussed.

Micro- or semimicromethods for the accurate determination in proteins of total sulfur (8, 9) and in protein hydrolysates of sulfate sulfur (10), cysteine (11, 12), cystine (11, 12), cysteine + cystine (10), methionine (10), tyrosine (13), tryptophane (13), and arginine (14) have been described in recent publications from this laboratory. A system of analysis has been developed in which most of these constituents are determined by two independent methods. Hydrolysis is carried out in an inert atmosphere with HI in presence of hypophosphite; with HCl, and with HCl in presence of urea (12);² with NaOH, and with NaOH-SnCl₂ in sealed tubes containing a minimum of enclosed air (13).

In HI digests, we determine methionine as volatile iodide and as homo-

¹ We are indebted to Professor Cannan for a sample of recrystallized β -lactoglobulin and for information in advance of publication (6, 7).

² Also Brand and Kassel, unpublished results.

TABLE I
Analysis of β -Lactoglobulin

Determination No. (1)	Constituent (2)	Hydrolysis (3)	Method (bibliographic reference Nos.) (4)	Per cent (5)	Concen- tration $10^3 \times C$ <small>$10^3 \times$ moles per gm. of atoms per gm.</small> (6)
1*	Cysteine	HCl-urea	(11, 12)	1.10	9.2
2*	Cystine	"	(11, 12)	2.29	19.1
3*	Cysteine + cystine	HI	(10)	3.39	28.3
4	Methionine	"	(10)	3.22	21.6
3	(Cysteine + cystine) S			0.905	28.3
4	Methionine S			0.692	21.6
5 = 3 + 4				1.597	49.9
6	Sulfate S	HI	As H ₂ S (10)	0	
7	Total S		Pregl (8, 9)	1.604	50.0
8 = 7 - 6	Protein S			1.604	
9 = $\frac{5+8}{2}$	Average pro- tein S			1.60	49.95
10	Tyrosine	NaOH-SnCl ₂	(13)	3.78	20.9
11	Tryptophane	"	(13)	1.94	9.5
12	Arginine	HCl	(14)	2.87	16.5
13†	Histidine†	"	Cf. (6)	2.0	13.0
14†	Lysine	"	" (6)	10.7	73.0
15	Threonine	"	(16, 17)	5.85	49.1
16	Total N		Pregl-Dumas	15.60	1113.6
17†	Amino "		Titration, Van Slyke (cf. (6))	1.23	88.0
18†	Amide "	HCl, NaOH	Cf. (6, 7)	1.08	77.0
11a	Indole "			0.13	9.5
12a	Guanidino N			0.69	49.2
13a	Imidazole N			0.36	26.0
14a	ϵ -Amino N			1.02	73.0
19 = 18 + 11a + 12a + 13a + 14a	Non- α -N			3.28	234.2
20 = 16 - 19	α -N			12.32	879.4
21 = 17 - 14a	Terminal α -N			0.21	15.0
22 = 20 - 21	Peptide N			12.11	864.6
23	Average residue weight = $1/C_{\alpha-N} = 113.7$				
23a	" " "	= $113.5\frac{1}{2} - 115.4\frac{1}{2}$ = $1/C_{\text{peptide N}} = 115.6$			

* Calculated as half cystine (mol. wt. = 120).

† Determinations by Cannan *et al.* (6, 7).

TABLE I—*Concluded*

† Average value; cf. foot-note 4.

§ These values result from the further evaluation of the data in Table II, Column 4.

cysteine, cysteine + cystine as cysteine, sulfate sulfur as H_2S (10). In HCl hydrolysates, preferably in the presence of urea (12),² cysteine and cystine are determined separately by our photometric method (11). The formation of acid-insoluble humin interferes with the cysteine determination, since the precipitate may contain appreciable amounts of cysteine, as was first shown by Lugg (15). By carrying out the HCl hydrolysis in the presence of urea, the formation of an acid-insoluble humin precipitate can be almost entirely prevented with certain carbohydrate-containing proteins such as egg albumin and lactalbumin, although the hydrolysate may be dark brown owing to acid-soluble humin. With carbohydrate-free proteins, the HCl-urea hydrolysates are usually perfectly clear and colorless, or at most slightly yellowish (12).² In such (urea-containing) HCl hydrolysates, satisfactory results for both cysteine and cystine are obtained. In NaOH and NaOH-SnCl₂ hydrolysates, tyrosine is quantitatively separated from tryptophane, which is isolated as an insoluble mercury compound. The Millon reaction is used for the photometric determination of both these amino acids (13).

The analytical results are reported in Table I. The average values for the various constituents are given in per cent in Column 5 and in terms of their concentration in moles (or atoms) per gm. in Column 6, where for convenience the actual values are multiplied by 10^5 . The molar concentration (C_i) of an individual amino acid (i) is defined by Equation 1,

$$C_i = \frac{(\%)_i}{100 \times M_i} \quad (1)$$

where M_i is the molecular weight of an individual amino acid (i) and $(\%)_i$, the content of (i) in per cent by weight in the dry, ash-free and salt-free protein. It is customary to report the results of protein analysis as per cent of amino acid, notwithstanding the fact the amino acids are present in peptide linkage as amino acid residues (minus H_2O). From Equation 1 it is obvious that the molar concentration is the same, irrespective of whether it is calculated from the per cent amino acid divided by the molecular weight or from the per cent residue divided by the residue weight. This also holds for the calculation of M_{min} according to Equations 3 and 4.

It can be seen from Table I that β -lactoglobulin contains 3.39 per cent of cysteine + cystine, one-third being cysteine and two-thirds cystine.³

³ Native β -lactoglobulin has no reactive SH groups, since we find that the nitroprusside test is negative (in the presence of ammonia and NaOH) and no oxidation

Since sulfate S is absent (Determination 6), all of the total S, determined by elementary analysis, is protein S (Determinations 7, 8). Methionine (3.22 per cent) together with cysteine and cystine accurately accounts for the protein sulfur (Determination 8). The average value for protein sulfur (Determination 9) has, therefore, a high degree of accuracy, the agreement being better than 1 per cent. The tyrosine and tryptophane content are 3.78 and 1.94 per cent respectively. The arginine content is 2.87 per cent by the method described in the preceding paper (14). Threonine (5.85 per cent) was determined by Winnick's adaptation to Conway vessels (16) of the method of Shinn and Nicolet (17). The value for total N (15.60 per cent) was obtained by the Pregl-Dumas method.

Included in Table I are values for lysine, histidine,⁴ amino N, and amide N recently reported by Cannan, Palmer, and Kibrick (6) and by Warner and Cannan (7). The nitrogen of the side chains (*i.e.* indole, guanidino, imidazole,⁴ and ϵ -NH₂-N) was calculated from the percentages of the corresponding amino acids (Determinations 11a to 14a). These values, together with the amide N, constitute the non- α -nitrogen (Determination 19), which subtracted from the total N gives the α -N (Determination 20 = 12.26 per cent N); *i.e.*, the average α -N content of the constituent amino acids, whether they are present in N peptide linkage or not. An estimate of the non-peptide (*terminal*) α -N can be obtained from the difference between the amino N (determined by titration and the Van Slyke procedure, Determination 17) and the ϵ -N of lysine (Determination 14a), on the assumption that neither proline nor hydroxyproline occupies a terminal position. This difference (*cf.* "Discussion" (6)) is rather large (Determination 21) and amounts to about 20 per cent of the ϵ -N of the lysine; it would indicate four to six such *terminal* groups for minimum molecular weights of from 30,000 to 40,000. Since the results for lysine by isolation procedures are apt to be low and since the value for amino N is probably accurate (titration and Van Slyke values check), the value for *terminal* α -N is prob.

by porphyrindin takes place (at 0° and pH 7). The heat-denatured protein, however, gives a strong nitroprusside test. The appearance of CyS—H groups upon heat denaturation and after acid hydrolysis may be due to the presence in the native protein of unreactive CyS—II or of CyS—X groups or both (for discussion *cf.* (12)). The preliminary experiments with porphyrindin also indicate that the phenolic groups are unreactive in the native protein.

⁴ A histidine content of 2.3 per cent was obtained by Cannan *et al.* (6) by the nitranilate method and from titration data; however, these authors could isolate only 1.7 per cent of histidine as flavianate (R. K. Cannan, private communication). In Table I (Determination 13) an average value of 2.0 per cent is given, corresponding to 5 residues in Table II. The effect of this uncertainty in the histidine content on Determinations 19, 20, 22, and 23 in Table I is slight, resulting in a possible error of ± 1 residue in the corresponding values in Table II.

ably too high. Therefore the value for α -N (Determination 20) represents a maximum value⁴ and that for peptide N (Determination 22, *i.e.* α -N less *terminal* α -N) a minimum.⁴ The average residue weight (*ARW*) of the constituent amino acids is the reciprocal of the concentration (in atoms per gm.) of the α -N.

$$ARW = 1/C_{\alpha-N} \quad (2)$$

The reciprocal of the concentration of peptide N will closely approximate the average residue weight if the number of *terminal* α -N groups is small. In view of the uncertainty in the *terminal* α -N, a definite value for the average residue weight of β -lactoglobulin (Determinations 23 and 23a) cannot be established, the range according to the present data being from 113.5 to 115.6 (for these figures the further evaluation of the data in Table II, Column 4, is taken into consideration).

From the data in Table I the minimum molecular weight ($M_{min.}$) of β -lactoglobulin can be calculated on the assumption that the material analyzed is a pure chemical individual and that its indicated molecular composition must therefore involve integral numbers of residues of the constituent amino acids. $M_{min.}$ of a protein is given by Equation 3,

$$M_{min.} = \frac{M_i \times R_i}{(\%)_i} 100 \quad (3)$$

where M_i and $(\%)_i$ are as previously defined and R_i is an integer and represents the number of residues of (*i*) per $M_{min.}$ Combining Equations 1 and 3 leads to Equation 4,

$$M_{min.} = R_i/C_i \quad (4)$$

If a single amino acid (*a*) has been determined, $M_{min.}$ is obtained from Equation 3 or 4 by taking $R_a = 1$. If two amino acids (*a*) and (*b*) have been determined, minimum values for R_a and R_b are obtained by setting up two simultaneous equations for $M_{min.}$, yielding Equation 5,

$$C_a/C_b = R_a/R_b \quad (5)$$

Conversion of this *common* fraction into the smallest *simple* fraction (*i.e.* one in which both numerator and denominator are integers) yields minimum values for R_a and R_b . For R_a a value of 1, 2, 3, etc., is taken successively until for R_b the corresponding smallest integer is found, from which the experimental data do not differ significantly. If additional amino acids (*c*), (*d*), etc., have been determined, Equation 5 is set up for individual pairs and the combined minimum ratio established. *E.g.*, if

$$\frac{R_a}{R_b} = \frac{3}{4}, \quad \frac{R_c}{R_d} = \frac{4}{9}, \quad \frac{R_a}{R_d} = \frac{1}{1}$$

the smallest integral ratio $a:b:c:d$ is 9:12:4:9. From the values for R so obtained and the corresponding concentrations, C , $M_{\min.}$ is calculated according to Equation 4.

Cystine (CyS—SCy, mol. wt. = 240) is the equivalent of two amino acids; it has, therefore, to be considered in terms of half cystine residues (CyS—, mol. wt. = 120); so that by definition the number of half cystine residues ($R_{\text{half cystine}}$) in a protein must always be an even integer. Cysteine (CyS—H, mol. wt. = 121) is best calculated in per cent of half cystine (mol. wt. = 120), and the number of cysteine residues in a protein molecule (R_{cysteine}) may obviously be even or odd.

At present, owing to experimental limitations on the accuracy of the analytical methods, significant results by this method of calculation can be obtained only for values of $M_{\min.}$ up to about 50,000. For higher values of $M_{\min.}$ such calculations become less and less significant, but under special conditions the limit may be extended to about 70,000. Amino acids present in very low or very high concentrations are not suitable for the calculations; the useful range of C_i is from 1.5 to 60×10^{-5} mole per gm. and the optimum is 10 to 40×10^{-5} mole per gm. The calculations must be restricted to amino acids which can be determined with a sufficient degree of accuracy (about 2 per cent). In the calculation of the integral values for the pairs of residues according to Equation 5, all values which differ from the nearest integers by more than about 2 per cent should be eliminated, while any value within 2 per cent of the nearest integers should be considered as a possible fit.

It is generally recognized that some uncertainty is attached to all amino acid determinations, since they are usually carried out in hydrolysates. The study of the behavior and destruction of the free amino acids under varying conditions (*cf.* (12) and foot-note 2) and the establishment of correction factors overcome this difficulty to some extent. There always remains the possibility that in a specific protein structure an amino acid is unusually labile and subject to increased hydrolytic destruction and other reactions. For example, the cystine in insulin is sensitive towards HI hydrolysis, whereas it is quite stable during HCl hydrolysis (18); also tryptophane in chymotrypsinogen is more extensively decomposed during hydrolysis by alkali alone than it is by alkaline stannite (12). However, in the case of the sulfur amino acids (and iodo amino acids), the analysis of hydrolysates may yield unequivocal evidence. If the protein sulfur, which can be determined by elementary analysis with an error of less than 1 per cent, is fully accounted for by methionine, cysteine, and cystine in hydrolysates, then we have conclusive evidence that the amount of the sulfur amino acids actually present in the protein has been determined. For the following proteins we have accurately accounted for the total

sulfur (usually within 1 per cent), casein, lactalbumin, reduced lactalbumin, crystalline egg albumin (8); thyroglobulin (19); cattle glohin, cattle blood fibrin;² chymotrypsinogen (12); horse serum albumin B (20); horse serum albumin A, human serum albumin, ribonuclease, pepsin, α -, β -, and γ -chymotrypsin, trypsinogen, and trypsin.²

In all these cases the number of sulfur atoms per $M_{\min.}$ equals the sum of the residues of the sulfur amino acids as given in Equation 6,

$$R_{\text{sulfur}} = R_{\text{methionine}} + R_{\text{cysteine}} + R_{\text{half cysteine}} \quad (6)$$

The values for the sulfur amino acids are quantitatively more significant than those obtained for other amino acids and afford a favorable basis for the calculation of $M_{\min.}$ according to Equations 3 to 6.

This approach has been satisfactory in the case of chymotrypsinogen (12) for which from the consideration of the sulfur distribution alone a value for $M_{\min.}$ was obtained which was practically identical with the molecular weight deduced from osmotic pressure (21). In the case of β -lactoglobulin, other amino acids have to be taken into consideration to obtain a significant figure for $M_{\min.}$

Using the concentrations in Table I, Column 6, for the calculation of R_a and R_b according to Equation 5, we find the following relationships for methionine and cysteine + cystine (the per cent deviation from the nearest integers is given in parentheses),

$$\frac{R_{\text{methionine}}}{R_{(\text{cysteine} + \text{half cysteine})}} = \frac{1}{1.31} (31\%), \frac{2}{2.62} (13\%), \frac{3}{3.93} (2\%), \frac{4}{5.24} (5\%),$$

$$\frac{5}{6.55} (6\%), \frac{6}{7.86} (2\%), \frac{7}{9.17} (2\%), \frac{8}{10.5} (5\%), \frac{9}{11.8} (2\%), \frac{10}{13.1} (1\%), \frac{11}{14.4} (2\%)$$

This series covers values for $M_{\min.}$ up to 50,000. It can be seen that for 3, 6, 7, 9, 10, and 11 residues of methionine, the corresponding residues of cysteine + half cystine (*i.e.* 4, 8, 9, 12, 13, and 14) are within 2 per cent of the nearest integer. The number of residues of cysteine + half cystine must be consistent with the number of residues of cysteine (R_{cysteine}) and of half cystine ($R_{\text{half cysteine}}$) derived from the separate determinations of these constituents (Table I, Determinations 1 and 2). Calculated³ according to Equation 5, $R_{\text{cysteine}}:R_{\text{half cysteine}} = 1:2$; so that only values which are multiples of 3 can be considered for $R_{(\text{cysteine} + \text{half cysteine})}$. This eliminates all but 9 and 12 from the series of figures given above. From the distribution of the sulfur amino acids, therefore, two alternative values are obtained for $M_{\min.}$ of β -lactoglobulin and both these values are equally

³ The ratio is 1/2.03; the deviation from the integer is 4 per cent; indeed a greater deviation would not necessarily be significant, because any cysteine that has been oxidized is accounted for as cystine.

consistent with the analytical data: (a) $M_{\min.} = 42,000$ with 9, 12, and 21 residues of methionine, cysteine + half cystine, and sulfur, respectively; (b) $M_{\min.} = 32,060$ with 7, 9, and 16 residues, respectively.

As pointed out above, the most accurate determination is that of the protein sulfur; so that the decision between 16 and 21 atoms of sulfur per $M_{\min.}$ must be made with the aid of additional data. Calculated according to Equation 5, $R_{\text{sulfur}} : R_{\text{tryptophane}}$ is 21:3.99 and 16:3.05, respectively; no decision can be made, since both figures are within 2 per cent of the nearest integers. For tyrosine we calculate (a) 21:8.78 (2.4 per cent) and (b) 16:6.70 (4.3 per cent); again no decision can be made, since both figures for the number of tyrosine residues differ by more than 2 per cent from integers. In view of the accurate accounting of the sulfur and of the close agreement of the tryptophane determination with the sulfur partition, we conclude that the tyrosine value is insufficiently accurate and is probably too low.

Next we try arginine and calculate $R_{\text{sulfur}} : R_{\text{arginine}}$ as 21:6.93 (1.0 per cent) and 16:5.29 (5.8 per cent). This favors (a).

In Table II the number of residues of the various constituents is given for $M_{\min.} = 42,000$. The experimentally found residue numbers (Column 3) are corrected to the nearest integers in Column 4, the deviation from these integers being noted in Column 5.

The total number of N atoms per mole is 468. This figure is accurate within a few N atoms, since it is based on the determination of the total N (15.60 ± 0.02 per cent) which can be considered as accurately established.⁶

Calculation of the total number of amino acid residues per mole ($R_{\text{total AA}}$, Determination 20 = No. 16 less No. 19) yields 370 including six *terminal* amino acids (terminal AA). Because of the uncertainty in the lysine and histidine⁴ values (cf. discussion of Table I) these figures represent maximum values; so that $R_{\text{total AA}}$ in β -lactoglobulin = $364 (\pm 3) +$ one to six *terminal* AA.

According to certain current views on protein structure *terminal* amino groups may be interpreted as indicating polypeptide chains. It is therefore possible that β -lactoglobulin may contain more than one polypeptide chain per mole, but this question cannot be decided until the lysine content has been accurately established.

⁶ Palmer (1) originally reported a total N content of 15.3 per cent for β -lactoglobulin. Later, however, he found 15.6 per cent by using a copper-mercury catalyst in the Kjeldahl determination (5). The Pregl-Dumas determination yields the same value (Table I, Determination 16), which accurately represents the N content of dry, ash- and salt-free β -lactoglobulin since it has been consistently found with a number of preparations in several laboratories (personal communication from R. K. Cannan).

β -Lactoglobulin has been studied in great detail (more than 70 runs are reported) in the ultracentrifuge in Svedberg's laboratory (2, 4). From sedimentation velocity and diffusion data and a specific volume of 0.751 (a value which we confirm), Pedersen computes an average value for M_s ,

TABLE II

Amino Acid Residues and Nitrogen and Sulfur Atoms per Mole of β -Lactoglobulin

$$M = M_{\min.} = M_s = 42,000.$$

Determination No., Table I	Constituent	No. of residues or atoms (R) per mole		
		Found (3)	Corrected (4)	Deviation* (5)
				<i>per cent</i>
9	Average protein S	20.98	21	0.1
3	Cysteine + half cystine	11.89	12	0.9
4	Methionine	9.07	9	0.8
11	Tryptophane	3.99	4	0.3
12	Arginine	6.93	7	1.0
1	Cysteine	3.86	4	3.5†
2	Half cystine	8.02	8	0.3
10	Tyrosine	8.78	9	2.4
13‡	Histidine	5.46	5§	
14‡	Lysine	30.7	31	
15	Threonine	20.7	21	
16	Total N	467.8	468	
17‡	Amino "	37.0	37	
18‡	Amide "	32.3	32	
19	Non- α -N	98.3	98	
20	α -N	369.3	370	
21	Terminal α -N	6.3	6	
22	Peptide N	363.3	364	

Total No. of amino acid residues per $M_{\min.} = 364 (\pm 3) + 1$ to 6 terminal AA

$$* \text{ Deviation} = \frac{(4) - (3)}{(4)} \times 100.$$

† Cf. foot-note 5.

‡ Determinations by Cannan *et al.* (6, 7).

§ Cf. foot-note 4.

of 41,600 and from sedimentation equilibrium an average for M_s of 38,000 (cf. (4, 22)). Pedersen's value for M_s is practically identical with $M_{\min.}$

It can be concluded that the minimum molecular weight and the molecular weight in solution of β -lactoglobulin are identical (factor = 1), or, in other words, that β -lactoglobulin is monomolecular in solution, and that its molecular weight is close to 42,000.

The close agreement between $M_{\min.}$ and M_s is significant, since it probably indicates that some of the theoretical objections raised against M_s are either not valid or are of no practical consequence, at least in the case of β -lactoglobulin. Both $M_{\min.}$ and M_s refer to the molecular weight of the unhydrated molecule, M_s for reasons pointed out by Svedberg and Pedersen (22) and $M_{\min.}$ because our determinations are carried out on air-dried material corrected for moisture (*cf.* (12) and experimental part). Consistent results for the moisture content of powdered, air-dried proteins can be obtained by drying to constant weight in an oven at 110° or, preferably, *in vacuo* over P_2O_5 at 100° (12). Sometimes it is questioned whether such results correspond to the actual moisture content of the protein, since, it is argued, some moisture might have been retained or some anhydride formation might have occurred. However, our moisture determinations would appear to give reliable results, since in preliminary experiments with Dr. D. Rittenberg we have found the same moisture content in crystalline egg albumin: (a) by drying at 100° *in vacuo* over P_2O_5 and (b) at room temperature by determining the water content by the isotope dilution method (23), water containing an excess of the heavy oxygen isotope O^{18} (*cf.* (24)) being used as indicator.

The accurate estimation of the moisture content of proteins enters into the calculation of their molecular weight from x-ray measurements of the unit cell volume. Crowfoot's latest estimate (25) of the molecular volume of air-dried tabular crystals of β -lactoglobulin is 52,000 cu. Å., but, in the absence of specific data on the moisture content (presumably about 7 per cent) of the crystals and of their density, no estimate of the molecular weight was made. A molecular weight of 36,500 (not corrected for residual moisture) had previously been deduced by Crowfoot and Riley (26) on the basis of a unit cell volume smaller than that recently reported (25), and on the assumption that the density of the crystals was the same as that of insulin (measured in an organic medium; *cf.* (27)).

Attention should be called to the large number (at least forty-five) of hydroxyl groups per mole of β -lactoglobulin. About fifteen of these are in serine⁷ and twenty-one in threonine, as estimated by the excellent methods of Nicolet and Shinn (17, 28, 29); nine are in tyrosine and an unknown further number in hydroxyproline. This is not unusual⁸ since our data²

⁷ On the basis of the value reported by Nicolet and Shinn (28).

⁸ Our values for serine are still approximate, but the threonine content of the following pure crystalline proteins can be considered as fairly accurate: horse serum albumin A and B (35 residues per mole), human serum albumin (33 residues per mole), chymotrypsinogen (35 residues per mole; *cf.* (12)), α -chymotrypsin (11.3 per cent), β -chymotrypsin (10.4 per cent), γ -chymotrypsin (10.6 per cent), trypsinogen (5.2 per cent), trypsin (5.8 per cent), ribonuclease (9.0 per cent), swine pepsin (denatured, 9.5

on crystalline horse serum albumin A and B and on crystalline human serum albumin indicate a minimum of 80 hydroxyl groups per mole of 70,000. It is uncertain whether in the native protein these groups form hydrogen bridges or whether they exist in combined form and are set free only on hydrolysis.

In 1906, Emil Fischer (30) warned that experimental progress might be hindered by too exclusive an emphasis on the peptide linkage and pointed out that the hydroxyls of the hydroxy-amino acids are by no means "indifferent" groupings in the protein molecule. Fischer also suggested (30) that these hydroxyl groups might exist in proteins in the form of esters or ethers. Very few such groups have been found. Only one ether linkage is known: the thyroxine in thyroglobulin. The thio ethers lanthionine (*cf.* (31)) and cystathionine ((32), *cf.* (33)) have as yet not been found in native proteins. Phosphoric acid esters exist; *e.g.*, in casein where some of the aliphatic hydroxyl groups are so linked. The occurrence of esters of sulfuric acid, in view of the sulfate content of many purified proteins, has to be considered as possible. Their presence is strictly excluded only if a protein has been obtained free from sulfate as, for example, β -lactoglobulin, egg albumin, insulin, pepsin, and others. Esters with carboxylic acids are still purely hypothetical; a small number may exist, but the pertinent data are as yet not accurate enough to exclude or to establish this type of linkage in any protein.⁹

With regard to hydrogen bonding, it would seem from the phrases cited above that Fischer had anticipated some such effect. Like the sulfhydryl groups, the phenolic hydroxyl groups of native β -lactoglobulin (similar to egg albumin (34)) are highly unreactive. They cannot be titrated (6), they react with Folin's phenol reagent only in strongly alkaline solution, and they are not oxidized by porphyrindin.³ This contrast to the behavior of free tyrosine may reasonably be ascribed to intramolecular hydrogen bonding, akin to chelation. To judge from the available evidence for other compounds (*cf.* (35-37)) the aliphatic hydroxyl groups in the native proteins may also be involved in hydrogen bonding and thus contribute to the cohesion of the molecule, particularly by hydrogen bridges through water molecules. Such water molecules would be expected to exchange

per cent), purified elastin (about 2.7 per cent). We are indebted to Dr. H. Neurath for horse serum albumin A, to Mr. Manfred Mayer from this Department for B, to Dr. W. H. Stein and Dr. E. G. Miller, Jr., for the elastin, to Dr. F. E. Kendall for the human serum albumin, to Dr. R. M. Herriott for pepsin, and for the other crystalline enzymes to Dr. M. Kunitz.

⁹ At the present state of our knowledge caution should be exercised in assuming that free carboxyl groups are equal to the difference between total dicarboxylic acids and amide groups.

readily with water containing the heavy isotope O^{18} (cf. above). Obliteration of hydrogen bridges may be related to denaturation by dehydrating agents. The hypothesis of the rôle of the hydroxyl groups in the binding of water is in harmony with Perutz's view on hydration; i.e., "the disposition of water between structural units of the molecule in such a way as to cause an internal expansion" (38).

The surprisingly large number of hydroxyl groups which can be recognized in the side chains of protein molecules is bound to play an important part in the future development of hypotheses of protein structure and enzyme action.

EXPERIMENTAL

The sample of β -lactoglobulin employed in these studies was obtained from Dr. Cannan as a suspension in water, preserved with toluene. Most of the water was removed by centrifugation and the crystalline mass was dried over P_2O_5 in a vacuum desiccator at room temperature. The dry cake was finely powdered in an agate mortar and equilibrated in air until it reached constant weight (9 days). The air-dried material was used for all analytical procedures, the weights being corrected for moisture content. The moisture determinations were carried out as described previously (12); average moisture content = 7.01 per cent. The analytical data are reported in Table I.

Total Nitrogen—We are indebted to Mr. W. Saschek for this determination (No. 16), carried out by the Pregl-Dumas method.

Total Sulfur—We are indebted to Mr. W. Saschek for this determination (No. 7), carried out by a modification of the Pregl method (8, 9, 12). The ash was negligible, being less than 0.1 per cent.

Methionine—The value reported in Table I (Determination 4) is the average of four determinations with about 250 mg. of protein each; the same results were obtained by the volatile iodide and homocysteine titrations (10).

Cysteine, Cystine, and Cysteine + Cystine—For the separate determination of cysteine and cystine by the photometric method (11) about 100 mg. of protein were hydrolyzed for 16 hours with HCl-urea in an inert atmosphere, as described previously (12); the hydrolysates were practically colorless and there was no solid humin. The values reported (Determinations 1 and 2) are the average of three determinations; hydrolysis for 8 hours gave low results.

Cysteine + cystine (Determination 3) was determined in HI hydrolysates simultaneously with methionine (10).

For the cystine determination by the Sullivan method 200 mg. of protein were hydrolyzed under nitrogen gas for 16 hours with 6 N HCl. Owing to

the presence of cysteine in the hydrolysate, the results were high (for discussion cf. (12)), the "cystine" content being 3.89 per cent. This is additional evidence (cf. (12)) that the difference between total S and methionine S is accounted for by cysteine and cystine and not by any other sulfur-containing compound.

Tyrosine and Tryptophane—The values reported (Determinations 10 and 11) are the average of three determinations with 30 to 35 mg. of protein in NaOH-SnCl₂ hydrolysates.

Arginine—The value reported (Determination 12) was obtained by the method described in the preceding paper (14).

Threonine—About 40 mg. of protein were hydrolyzed under nitrogen for 16 hours at 130° with 2 cc. of 6 N HCl. The value reported (Determination 15) is the average of three separate determinations by Winnick's modification of Shinn and Nicolet's method. Winnick (16) reports a threonine content of 5.36 per cent for his preparation of β -lactoglobulin, which, however, contained only 14.35 per cent of nitrogen. Nicolet and Shinn (28) find only 4.72 per cent of threonine but state that, because of the small amount of material available for analysis, their value may be less reliable than some of their other determinations.

Specific Volume—The determination was carried out in capped pycnometers (about 10 cc.). The concentration was 1.816 per cent of β -lactoglobulin in 0.5015 per cent NaCl ($d_4^{25.6} = 0.99960$). The density of the protein solution was $d_4^{25.6} = 1.00410$. From these data we compute $V_{25.6} = 0.754$, corrected $V_{20} = 0.750$, which is in close agreement with Pedersen's (4) value of $V_{20} = 0.7514$.

SUMMARY

For β -lactoglobulin a minimum molecular weight of 42,000 was obtained from the distribution of the sulfur amino acids and from the arginine content.

The minimum molecular weight is practically identical with the molecular weight in solution ($M_s = 41,600$). Therefore, $M = M_{\min.} = M_s$. The total number of amino acid residues per molecule of β -lactoglobulin is estimated to be $364 (\pm 3) +$ one to six *terminal* amino acids.

1 molecule of β -lactoglobulin contains the following residues: cysteine 4, half cystine 8 (i.e. 4 S—S linkages), methionine 9, tryptophane 4, tyrosine 9, arginine 7, threonine 21, serine about 15, amide groups 32, histidine 4 to 6, and lysine 31 to 36.

Attention is called to the large number of hydroxyl groups which can be recognized in the side chains of β -lactoglobulin and of other proteins. The possible contribution of the hydroxyl groups to the cohesion of the molecule by hydrogen bridges through water molecules is discussed.

BIBLIOGRAPHY

1. Palmer, A. H., *J. Biol. Chem.*, **104**, 359 (1934).
2. Pedersen, K. O., *Biochem. J.*, **30**, 948 (1936).
3. Sørensen, M., and Sørensen, S. P. L., *Compt.-rend. trav. lab. Carlsberg, Series C*, **23**, 55 (1938).
4. Pedersen, K. O., *Biochem. J.*, **30**, 961 (1936).
5. Sørensen, M., and Palmer, A. H., *Compt.-rend. trav. lab. Carlsberg, Series C*, **21**, 283 (1938).
6. Cannan, R. K., Palmer, A. H., and Kibrick, A. C., *J. Biol. Chem.*, **142**, 803 (1942).
7. Warner, R. C., and Cannan, R. K., *J. Biol. Chem.*, **142**, 725 (1942).
8. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 435 (1938).
9. Saschek, W., *Ind. and Eng. Chem., Anal. Ed.*, **9**, 491 (1937).
10. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 145 (1938).
11. Kassell, B., and Brand, E., *J. Biol. Chem.*, **125**, 115, 131 (1938).
12. Brand, E., and Kassell, B., *J. Gen. Physiol.*, **25**, 167 (1941).
13. Brand, E., and Kassell, B., *J. Biol. Chem.*, **131**, 489 (1939).
14. Brand, E., and Kassell, B., *J. Biol. Chem.*, **145**, 359 (1942).
15. Lugg, J. W. H., *Biochem. J.*, **32**, 775 (1938).
16. Winnick, T., *J. Biol. Chem.*, **142**, 461 (1942).
17. Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138**, 91 (1941).
18. Kassell, B., and Brand, E., *Proc. Soc. Exp. Biol. and Med.*, **35**, 444 (1936).
19. Brand, E., Kassell, B., and Heidelberger, M., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **128**, p. xi (1939).
20. Brand, E., and Kassell, B., *J. Biol. Chem.*, **141**, 999 (1941).
21. Kunitz, M., and Northrop, J. H., *J. Gen. Physiol.*, **18**, 433 (1935).
22. Svedberg, T., and Pedersen, K. O., *The ultracentrifuge*, Oxford, table 48 (1940).
23. Rittenberg, D., and Foster, G. L., *J. Biol. Chem.*, **133**, 737 (1940).
24. Mills, G. A., and Urey, H. C., *J. Am. Chem. Soc.*, **62**, 1019 (1940).
25. Crowfoot, D., *Chem. Rev.*, **28**, 215 (1941).
26. Crowfoot, D., and Riley, D. P., *Nature*, **141**, 521 (1938).
27. Bull, H. B., Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **6**, 140 (1938).
28. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **142**, 139 (1942).
29. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).
30. Fischer, E., *Ber. chem. Ges.*, **39**, 530 (1906).
31. Horn, M. J., Jones, D. B., and Ringel, S. J., *J. Biol. Chem.*, **144**, 93 (1942).
32. Binkley, F., and du Vigneaud, V., *J. Biol. Chem.*, **144**, 507 (1942).
33. Brand, E., Block, R. J., Kassell, B., and Cahill, G. F., *Proc. Soc. Exp. Biol. and Med.*, **35**, 501 (1936).
34. Brand, E., and Kassell, B., *J. Biol. Chem.*, **133**, 437 (1940).
35. Pauling, L., *The nature of the chemical bond*, Ithaca (1939).
36. Mirsky, A. E., and Pauling, L., *Proc. Nat. Acad. Sc.*, **22**, 439 (1936).
37. Huggins, M. L., in Luck, J. M., Annual review of biochemistry, Stanford University, **11**, 27 (1942).
38. Perutz, quoted by Crowfoot (26).

DISSIMILATION OF PYRUVIC ACID BY CELL-FREE PREPARATIONS OF CLOSTRIDIUM BUTYLICUM*

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Proliferating cultures and resting cell preparations of the butyric anaerobes ferment pyruvic acid to yield acetic acid, carbon dioxide, molecular hydrogen, and occasionally other products (1-8). Existing evidence seems to indicate that the molecular hydrogen evolved in this fermentation and in the fermentation of glucose by these organisms may arise by the hydrogenlyase reaction from formic acid formed intermediately.

In this paper a cell-free preparation from *Clostridium butylicum* is described which converts pyruvic acid into acetic acid, carbon dioxide, and molecular hydrogen, but fails to act on formic acid.

EXPERIMENTAL

Methods

Medium and Culturing—The culture used in this work was *Clostridium butylicum* Strain 21 of the Wisconsin collection, a butyric anaerobe forming butyl and isopropyl alcohols. It was carried as a spore stock on soil.

The organisms were grown in 5 gallon bottles containing 16 liters of the following medium: 1.5 per cent glucose, 0.1 per cent Cuban blackstrap molasses, 0.125 per cent solubilized liver powder, 0.075 per cent Difco yeast extract, 0.05 per cent dibasic ammonium phosphate, 0.15 per cent ammonium sulfate, 0.005 per cent magnesium sulfate, and 0.0025 per cent manganese sulfate. The medium was adjusted to pH 6.8 to 7.0 and autoclaved for 1 hour at 120°. By sterilizing a solution of the ammonium salts separately, the amount of inert precipitate appearing during autoclaving could be minimized. The bottles were inoculated with 3 per cent of a 10 hour culture in 2 per cent glucose-0.5 per cent tryptone medium, and incubated at 37°.

This strain tends to form a heavy slime at about 18 hours. If harvested after that time, the cells are centrifuged with difficulty and have a high endogenous evolution of gas. The cells were therefore harvested while the culture was gassing actively, but before slime formation was excessive.

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Enzyme Preparations The production of the enzyme preparations used is illustrated by the following typical experiment. After 12 hours incubation the cells from 96 liters of medium were harvested by centrifuging in a Sharples supercentrifuge. The 159 gm. of wet cells (22.5 per cent dry matter) thus obtained were packed into stoppered tubes and immediately frozen. Such frozen cells lose about 20 per cent of their activity in 2 weeks. While frozen, the cells slowly rupture, and the cell contents are released. After 12 days freezing, 85 gm. of cells were suspended in sufficient freshly boiled, ice-cold water to make a thin cream (250 ml. volume), allowed to stand for about 10 minutes, and the cell debris was centrifuged down. The slightly cloudy, amber supernatant liquid was poured off. This liquid contained most of the activity of the original frozen cells. It was immediately frozen in a dry ice-acetone bath on the walls of several suction flasks and dried under a high vacuum. The gray dry material remaining weighed 5.28 gm. This vacuum-dried water extract was powdered and stored in a refrigerated desiccator. Before use it was suspended in freshly boiled water. It had a Q_{H_2} of 63 and a negligible endogenous evolution of gases. During storage for 3 months there was no loss of activity.

Preparation of Heated Liver Extract—Unless otherwise indicated, excess heated extract of fresh rat liver was added to the reaction mixtures. This was prepared by homogenizing fresh adult rat liver with an equal weight of water, heating in a boiling water bath for 5 minutes, centrifuging, and pouring off the active supernatant liquid.

Manometric Measurement of Gas Evolution—Except as indicated, the reactions were carried out in Warburg manometers under nitrogen at 37°. Evolution of carbon dioxide and hydrogen was measured independently by using sets of two manometers. The manometers contained identical reaction mixtures. To one, acid was added from a side arm at the completion of the experiment to insure complete liberation of dissolved carbon dioxide. This manometer measured the evolution of both carbon dioxide and hydrogen. The second, used to determine hydrogen evolution alone, contained KOH in the center cup. Carbon dioxide evolution was calculated by difference.

To measure enzyme activity, dilute solutions containing 12.5 to 50.0 micromoles of redistilled pyruvic acid, 125 micromoles of phosphate buffer (except as indicated), an excess (0.3 ml.) of freshly prepared heated extract of rat liver, and sufficient water to give a final liquid volume of 3.0 ml. were placed in the main compartment of Warburg flasks. The enzyme suspension was placed in a side arm. Except in a study of the effect of pH, all solutions were adjusted to pH 6.5. Yellow phosphorus was placed in the center cup or vacant side arm. After replacement of the atmosphere

with nitrogen, the reaction was begun by tipping the enzyme suspension into the main compartment, and the evolution of gases was followed. Endogenous gas evolution, in control flasks in which the pyruvate solution was replaced by water, was usually negligible or entirely absent.

When desired, residual pyruvate was determined manometrically by the ceric sulfate oxidation method of Silverman and Werkman (9).

Reaction Balance

A quantitative reaction balance of pyruvic acid fermentation by dried water extract of frozen cells is given in Table I. The reaction was carried out under nitrogen in a 25 × 200 mm. test-tube. As a control, a duplicate tube containing no pyruvate was used. Nitrogen was passed through the reaction mixture for several minutes before addition of the enzyme suspension. Carbon dioxide was absorbed in a known amount of KOH, and

TABLE I
Reaction Balance of Pyruvic Acid Fermentation

The reaction mixture contained 1.98 mM of sodium pyruvate, 450 mg. of dried water extract of frozen cells, 5.0 mM of phosphate, and 5 ml. of heated liver extract. pH 6.5. Total volume, 40 ml. Incubated under nitrogen at 37° for 5 hours.

Substance	Substance produced	Product per mole fermented pyruvate
	mM	moles
Pyruvic acid	-1.98	
Acetic " . . .	1.88	0.95
Carbon dioxide	2.01	1.01
Hydrogen	1.78	0.90

hydrogen was determined by water displacement. After 5 hours incubation, gas evolution had ceased and no residual pyruvate remained. To insure release of bound carbon dioxide, the reaction mixture was adjusted to pH 2.0 with sulfuric acid; dissolved carbon dioxide remaining in the solution was aspirated from an aliquot into KOH.

An aliquot of the reaction mixture was adjusted to pH 7.5. Volatile neutral products were removed by distillation of one-half the volume. In the distillate ethyl and butyl alcohols were determined by the method of Johnson (10), and acetone and acetylmethylcarbinol by a modified Mesinger titration. None of these substances was found to be present.

The remaining liquid was evaporated to a small volume and adjusted to pH 2.0 with sulfuric acid. Volatile acids were steam-distilled off and determined by a modified Duclaux method. Volatility constants of the acid showed that only acetic acid was present.

Lactic acid was determined by the method of Friedemann and Graesser (11) in an ether extract of an acidified aliquot of the reaction mixture. None was found. Apparently pyruvic acid is fermented to yield mainly acetic acid, molecular hydrogen, and carbon dioxide.

Effect of pH

The effect of pH on the rate of hydrogen evolution is shown in Fig. 1. Hydrogen evolution is most rapid at about pH 6.5.

The ratio of gases evolved at various pH levels was determined in a duplicate experiment. The ratio of carbon dioxide to hydrogen was 0.99 at pH 6.0 and 6.5, and 0.90 at pH 7.5. Reduction of hydrogen acceptors

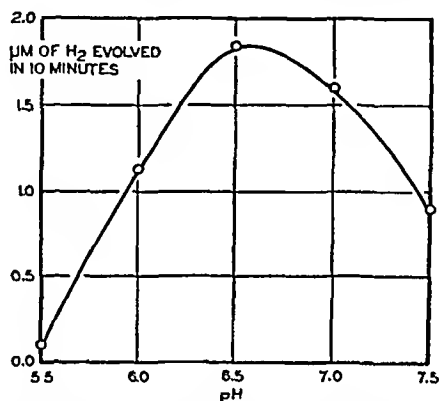


FIG. 1. Effect of pH on rate of hydrogen evolution. Each Warburg flask contained 12.5 micromoles of pyruvate and supernatant liquid from 70 mg. of suspended frozen cells. All solutions were adjusted to the proper pH. The rate of hydrogen evolution was constant for an hour.

present in the enzyme preparation, or a change in the type of reaction undergone by pyruvate, may account for the drop in the ratio at pH 7.5.

Specificity

The activity of the enzyme preparations on various substrates at pH 6.5 is given in Table II. The preparations fail to catalyze anaerobic evolution of gas from formic, succinic, lactic, or 3-phosphoglyceric acid, or glucose.

No hydrogenase activity could be detected with the use of large amounts of enzyme preparations by the Thunberg methylene blue reduction technique.

Effect of Phosphate Concentration

The effect of the concentration of added inorganic phosphate on the rate of hydrogen evolution is shown in Fig. 2. In this experiment sufficient

buffer capacity to maintain pH 6.5 ± 0.1 in the absence of added buffer was provided by the enzyme preparation.

TABLE II
Activity of Enzyme Preparations on Various Substrates

Each Warburg flask contained 50 micromoles of substrate, 0.3 ml of heated extract of fresh rat liver, and 30 mg. of vacuum-dried water extract of frozen cells. pH 6.5.

Substrate	Gas evolved in 30 min.*
	micromoles
None	0.28
Pyruvic acid	29.20†
Formic "	0.18
dl-Lactic acid	0.36
Glucose	0.12
3-Phosphoglyceric acid	0.12
Succinic acid	0.12

* Calculated as equimolecular evolution of hydrogen and carbon dioxide.

† Hydrogen evolution only.

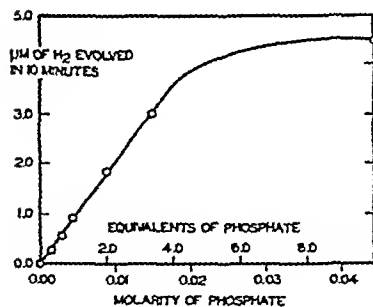


FIG. 2

FIG. 2 Effect of added phosphate on rate of hydrogen evolution. Each flask contained 12.5 micromoles of pyruvate, supernatant liquid from 63 mg of frozen cells, and added inorganic phosphate as shown. On the scale of phosphate equivalents, 1 equivalent is 12.5 micromoles.

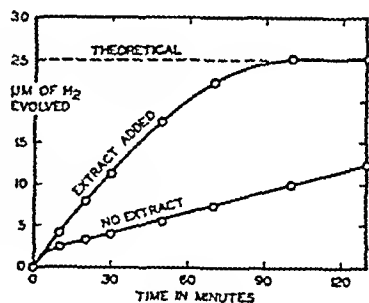


FIG. 3

FIG. 3 Activation by heated extract of fresh liver. Each flask contained 25 micromoles of pyruvate, 100 micromoles of phosphate, supernatant liquid from 67 mg of suspended frozen cells, and 0.5 ml of heated rat liver extract where indicated.

The rate of hydrogen evolution is proportional to the concentration of added phosphate up to 0.02 M. Although the heated liver extract and the enzyme preparation contain a total of 3.1 micromoles of apparent inorganic phosphate as determined by the method of Fiske and Subbarow (12), there

is no reaction unless inorganic phosphate is added. It will be noted from Fig. 2 that about 5 times as much phosphate are required for maximum reaction velocity as would be utilized by a phosphorylation reaction.

No stable phosphorylation product accumulates during the reaction.

Activators

Preparations of the enzyme show a pronounced dilution effect. That is, doubling the enzyme concentration more than doubles the reaction rate. Addition of heated, deproteinized water extract of either the supernatant liquid from a suspension of frozen cells or of homogenized fresh liver results in a several fold increase in reaction rate. The effect of adding heated extract of fresh liver on the rate of hydrogen evolution is shown in Fig. 3.

Attempts to replace the heated liver extract by adding 5 γ each of co-carboxylase, muscle adenylic acid, flavin-adenine dinucleotide, and co-enzyme I, and 10^{-3} M Mg^{++} and 10^{-4} M Mn^{++} , or combinations thereof, were unsuccessful. These substances had no effect on the reaction rate.

DISCUSSION

The enzyme preparations described catalyze the anaerobic dehydrogenation and decarboxylation of pyruvic acid to yield acetic acid, carbon dioxide, and molecular hydrogen. These preparations vary from preparations of previous workers (5-8) in that they are cell-free, and do not produce other products.

The origin of molecular hydrogen in the butyric fermentation is unknown. Donker (13) suggested that formic acid was produced intermediately and split into hydrogen and carbon dioxide; he noted increases in hydrogen evolution in the fermentation of glucose by *Clostridium beijerinckii* Donker when calcium formate was added. Stiles, Peterson, and Fred (14), however, found that added formate was fermented only very sluggishly in a corn mash fermentation by *Clostridium acetobutylicum*. While formic acid production in normal butyric fermentations of glucose is questionable (early reports of the production of minute amounts are probably erroneous because of the failure to remove acetylmethylcarbinol from test solutions before oxidation with mercuric chloride in the determination of formic acid), Osburn, Brown, and Werkman (15) were able to accumulate up to 0.47 mole of formic acid per mole of glucose fermented by *Clostridium butylicum* by maintaining a relatively alkaline pH during the fermentation. However, the failure of hydrogen-producing cell-free preparations to act on formic acid suggests strongly that formic acid is not an intermediate in the production of hydrogen.

The fermentation of pyruvic acid by these preparations appears to be somewhat similar to the oxidation of pyruvic acid catalyzed by the enzyme systems prepared by Lipmann (16) from cells of *Lactobacillus delbrueckii*.

It differs in the nature of the ultimate hydrogen acceptor. In the oxidation of pyruvic acid, molecular oxygen is reduced to hydrogen peroxide, while in the fermentation by preparations from *Clostridium butylicum* molecular hydrogen is formed.

The function of heated liver extract in the activation of our enzyme preparations is being investigated further. Lipmann (16) has found that cocarboxylase, flavin-adenine dinucleotide, inorganic phosphate, and divalent metals are required for the oxidation of pyruvic acid by his preparations. Combinations of these are required in the catalysis of other microbiological reactions involving pyruvic acid (9, 17). Apparently some additional factor may be necessary for the fermentation of pyruvic acid by our preparations, since the addition of these and other possible activators was without effect.

The rôle of inorganic phosphate in the reaction is also being investigated further. Although an uptake of inorganic phosphate has not yet been demonstrated, an intermediate phosphorylation is indicated. From approximations given by Kalckar in a recent review (18) of the free energy change in the reaction, it can be calculated that the energy available probably permits one phosphorylation per mole of pyruvic acid utilized. It is possible that a labile intermediate phosphate ester, such as acetyl phosphate, isolated by Lipmann in the oxidation of pyruvic acid (19), is produced.

SUMMARY

1. The preparation of a vacuum-dried cell-free water extract from frozen cells of *Clostridium butylicum* is described. This extract catalyzes the fermentation of pyruvic acid to acetic acid, carbon dioxide, and molecular hydrogen.

2. The reaction is most rapid at pH 6.5.

3. The dried preparations catalyze the anaerobic evolution of gases at pH 6.5 from pyruvic acid, but not from formic, lactic, succinic, or 3-phosphoglyceric acid, or glucose.

4. The rate of hydrogen evolution from pyruvic acid is proportional to the concentration of added inorganic phosphate up to 0.02 M. No stable phosphorylation product accumulates.

5. Preparations of the enzyme show a pronounced dilution effect, which can be overcome by adding heated extract of fresh liver or of frozen cells. This cannot be replaced by coenzyme I, cocarboxylase, flavin-adenine dinucleotide, muscle adenylic acid, or divalent magnesium or manganese.

BIBLIOGRAPHY

1. Neuberg, C., and Arinstein, B., *Biochem. Z.*, **117**, 269 (1921)
2. Langlykke, A., Peterson, W. H., and Fred, J. B., *J. Bact.*, **34**, 443 (1937).
3. Johnson, M. J., Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, **101**, 145 (1933)

4. Bernhauer, K., Iglauer, A., Groag, W., and Köttig, R., *Biochem. Z.*, **287**, 61 (1936).
5. Osburn, O. L., Brown, R. W., and Werkman, C. H., *Iowa State Coll. J. Sc.*, **12**, 275 (1937).
6. Brown, R. H., Osburn, O. L., and Werkman, C. H., *Proc. Soc. Exp. Biol. and Med.*, **36**, 203 (1937).
7. Kubowitz, F., *Biochem. Z.*, **274**, 285 (1934).
8. Davies, R., and Stephenson, M., *Biochem. J.*, **35**, 1320 (1941).
9. Silverman, M., and Werkman, C. H., *J. Biol. Chem.*, **138**, 35 (1941).
10. Johnson, M. J., *Ind. and Eng. Chem., Anal. Ed.*, **4**, 20 (1932).
11. Friedemann, T. E., and Graesser, J. B., *J. Biol. Chem.*, **100**, 291 (1933).
12. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
13. Donker, H. J. L., Thesis, Technische Hoogeschool, Delft (1926).
14. Stiles, H. R., Peterson, W. H., and Fred, E. B., *J. Biol. Chem.*, **84**, 437 (1929).
15. Osburn, O. L., Brown, R. W., and Werkman, C. H., *J. Biol. Chem.*, **121**, 685 (1937).
16. Lipmann, F., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 248 (1939).
17. Still, J. L., *Biochem. J.*, **35**, 380 (1941).
18. Kalckar, H. M., *Chem. Rev.*, **28**, 71 (1941).
19. Lipmann, F., *Federation Proc.*, **1**, pt. 2, 122 (1942).

DETERMINATION OF AMINO ACIDS IN PLASMA BY THE NINHYDRIN-CARBON DIOXIDE REACTION WITHOUT REMOVAL OF PROTEINS

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The present paper describes a method which has been in use for the past 3 years by the writer (1) and a number of colleagues to whom it was personally communicated (2). It is an application of the ninhydrin- CO_2 method of Van Slyke, Dillon, MacFadyen, and Hamilton (3) for determining free amino acids.

The specificity of the ninhydrin- CO_2 reaction for α -amino acids makes possible the direct analysis of plasma without removal of the proteins. During the 10 minute heating period used for the reaction small amounts of CO_2 are evolved from the proteins and the urea of the plasma, but the amounts of this CO_2 are so consistent that corrections can be made for them.

The amino acid content of serum formed by clotting of either whole blood or plasma has been found to be definitely higher than the content of plasma. The process of clotting is accompanied by a marked increase in the free amino acids of the fluid. Results of serum analyses cannot therefore be used as accurate measures of the amino acid content of the circulating plasma.

In the general technique of "submicro" (3) manometric analyses by the ninhydrin- CO_2 method, with such small amounts that the manometer readings are made with the CO_2 gas at 0.5 cc. volume, an improvement has been introduced by nearly saturating with NaCl the 0.5 N NaOH and 2 N lactic acid which are introduced into the chamber. Thereby the i correction of Van Slyke and Neill (4), for reabsorption of CO_2 while the meniscus is raised to the 0.5 cc. mark, is diminished from 3.7 to 0.6 per cent, and errors from its variation are lowered to less than 1 part per 1000 of the CO_2 determined.

Also a procedure has been found for treating rubber connections in such a way as to minimize their uptake and giving off of traces of CO_2 , and to render possible the use of a rubber-stoppered Erlenmeyer flask as a reaction vessel, even when, as in plasma analyses, the sample contains only 30 to 50 γ of carboxyl nitrogen.

Apparatus

The apparatus is the same as that described by Van Slyke, Dillon, MacFadyen, and Hamilton (3), with the exception that a convenient adaptation of stock 25 cc. Pyrex Erlenmeyer flasks as reaction vessels has been em-

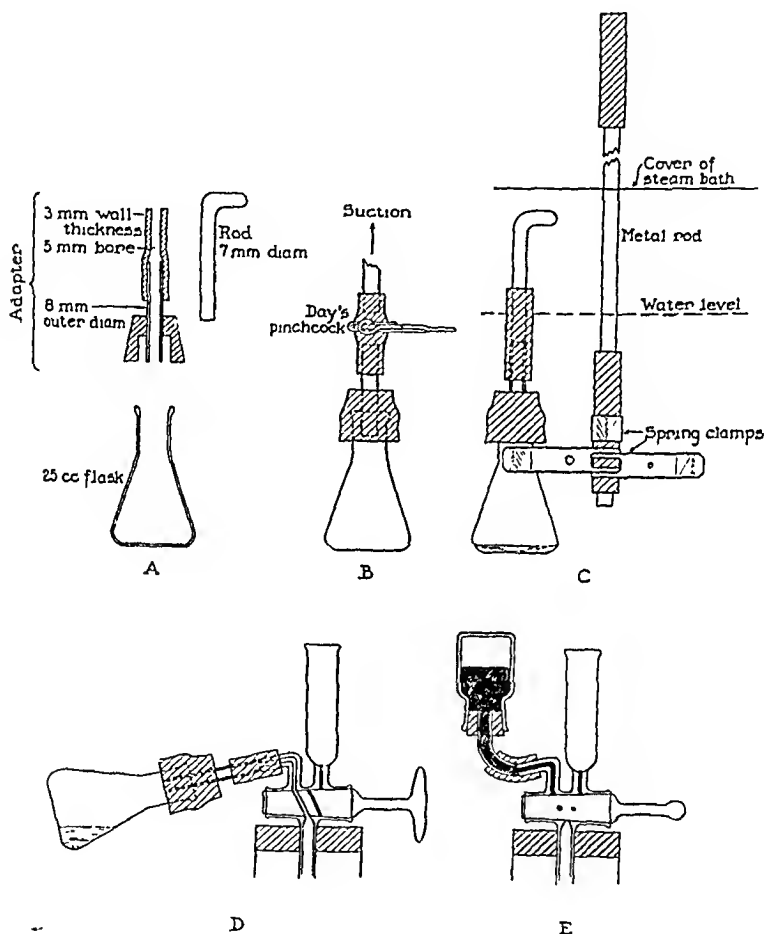


FIG. 1. A, parts of the reaction flask. B, temporary clamping of tube after evacuation of the flask. C, clamp replaced by glass rod; vessel attached to the rack in the steam bath. D, attachment of reaction vessel to the Van Slyke-Neill chamber. E, use of mercury bottle to seal the cock of the Van Slyke-Neill chamber.

ployed. Also a simple rack has been devised for holding the reaction vessels in an ordinary steam bath, and a convenient form of mercury bottle for sealing the Van Slyke-Neill chamber.

Erlenmeyer Flask Reaction Vessel—Pyrex Erlenmeyer flasks with flanges

as they are obtained ordinarily make suitable reaction vessels. If the flange is not circular, it is made so by grinding the uneven edge until it fits tightly into the adapter.

The flask is provided with an adapter (Fig. 1, *A*) which serves to close the flask during the reaction and then to connect it with the Van Slyke-Neill chamber. The adapter consists of a glass tube of 8 mm. outer diameter and 4 to 5 cm. long, bearing an equal length of rubber tube at one end and a No. 6 rubber stopper at the other. The rubber tube has a bore of 5 mm. (3/16 inch) and a wall thickness of 3 mm. (1/8 inch).¹ The stopper has a bore of 16 mm. for one-half its length and of 5 to 6 mm. for the rest of its length.² Before the rubber pieces are used, they are scrubbed with vaseline (not treated with NaOH, HCl, or water) to remove "bloom," and are wiped to remove excess of vaseline. During reactions the adapter is closed by a glass rod of 7 mm. diameter, rather than by a clamp on the tube, because the arrangement with the rod, as shown in Fig. 1, *C*, exposes less rubber surface.

Technique of Handling Reaction Vessel in Analyses—After the air in the flask has been evacuated, preparatory to an analysis (see p. 641 (3)), the adapter is first closed with a Day pinch-clamp, as shown in Fig. 1, *B*. The glass tube leading to the suction pump is then withdrawn and the glass rod (Fig. 1, *A*) is lubricated with glycerol and inserted while the tube above the clamp is pinched flat between the fingers to minimize the volume of air trapped between the rod and the clamp. (The desirability of minimizing the amount of air in the vessel is discussed on p. 642 of the original paper (3)). The pinch-clamp is then removed, and the glass rod is pushed further in until it is flush against the glass tube, as in Fig. 1, *C*. When the reaction has been finished, the flask is prepared for connection with the Van Slyke-Neill chamber by withdrawing the rod and at the same time replacing the pinch-clamp so that the vacuum in the flask is not released. The rubber tube above the clamp is then pinched between the fingers, the flask is connected to the chamber, the pinch-clamp removed, and the glass tube of the adapter pushed up against that of the chamber, as in Fig. 1, *D*.³

¹ We have used the red rubber tubing listed as "nitrometer tubing, A. H. T. specifications" No. 8842, in the Arthur H. Thomas Company's catalogue. We have also used the black rubber tubing listed as of "minimal sulfur content" No. 30691 of Eimer and Amend's 1936 catalogue.

² We have used rubber stopper, size No. 6, listed as HR-108 by Arthur H. Thomas Company. It comes channeled to one-half its length with the 16 mm. hole, and requires only the boring of the 5 to 6 mm. hole.

³ The same technique, with closure by a rod instead of a clamp, can be applied to the vessels shown in Fig. 1, *A* and *B* of Van Slyke, Dillon, MacFadyen, and Hamilton (3), so that the rubber area exposed in either of these vessels is contracted to two circular lines. In place of the glass vessel of Fig. 1, *C* of the above authors (3) Van Slyke and Hamilton (to be published) have introduced a simpler all-glass vessel which eliminates the need of precautions for "rubber CO₂;" its greater cost, compared with rubber-closed vessels, is justified when numerous analyses are to be done.

Rack for reaction vessels. The rack shown in Fig. 1, *C* is an easily improvised arrangement which permits one to sink the reaction vessels to the floor of a bath; so that the rather shallow steam baths found in stock equipment can be used for the reaction. The rack consists of a rod, preferably metal, sheathed at each end with rubber tubing, and provided with four spring elamps.⁴ The rubber sheaths serve two purposes; the spring elamps can be wired securely to the lower sheath; the upper sheath is cooler to handle than the exposed metal when the rack is lifted from boiling water.

Bottle containing mercury for sealing the curved capillary of Van Slyke-Neill extraction chamber. The device shown as *E* in Fig. 1 is convenient.

The bottle is first attached to the chamber, and then is rotated upwards to the position shown. The curved glass tube from the bottle has a bore of at least 6 mm.; so that when the bottle is rotated to the position shown in Fig. 1, *E*, air in the tube will be easily displaced upwards by mercury. Less than 0.1 cc. of air is admitted to the extraction chamber when the capillary is sealed in this way. Since 1 cc. of laboratory air may contain at times as much as 1 c.mm. of CO_2 , or enough to give 2 mm. pressure at 0.5 cc. volume, it is desirable to minimize admission of air in order to maintain constancy of the blank for microanalyses.

Solid glass rods for closing reaction vessel. The rod shown in Fig. 1, *A* and *C* is 6 cm. long, 7 mm. in diameter, and is bent at one end to facilitate withdrawal from the adapter.

Reagents

The reagents are the same as those used by Van Slyke, Dillon, MacFadyen, and Hamilton (3) with the following modifications.

Citrate buffer solution. It is convenient in the plasma analyses to use citrate buffer of pH 2.5 in solution instead of solid form. 20.6 gm. of trisodium citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) and 191 gm. of citric acid ($\text{C}_6\text{H}_8\text{O}_7 \cdot \text{H}_2\text{O}$) are dissolved in 1 liter of water. A few crystals of thymol are added as preservative.

0.5 N NaOH, approximately CO_2 -free, and 2.0 N lactic acid, both in nearly saturated salt solution are prepared as described on pp. 637-638 of Van Slyke *et al.* (3), with the exception that instead of water to dilute the concentrated NaOH and lactic acid, one uses a 25 per cent by weight solution of NaCl. The salt solution is prepared by dissolving 250 gm. of NaCl in 750 cc. of water. A few drops of 1 per cent alizarin red solution are added, and 1 N HCl until the indicator turns clear yellow. Traces of dissolved CO_2 are then removed by shaking the solution in a large evacuated vessel or bubbling a current of air through it.

⁴ Metal spring clamps of appropriate size and strength are sold by the Fisher Scientific Company, by E. Machlett and Son, and by the Arthur H. Thomas Company, as supports for their models of the Van Slyke-Neill manometer assembly.

Glycerol is used as a lubricant for the glass tube inserted as shown in Fig. 1, *E*. The glycerol should be neutral or slightly acid to prevent absorption of CO_2 .

Procedure

Preliminary Vacuum Heating of Adapter to Remove "Rubber CO_2 "—When the adapter (Fig. 1, *A*) stands in air for 2 or more days, CO_2 accumulates in the pores of the rubber and thence escapes into the evacuated, heated reaction vessel during the next analysis for which the adapter is used. The CO_2 from the adapter may be enough to increase the manometer reading by 12 to 15 mm., when the reading is taken with the gas at 0.5 cc. volume. However, the following treatment, carried out on the day of analysis, reduces this "rubber blank" to about 4 mm., and the adapter can be used for a series of successive analyses on the same day without changing the blank by more than 0.5 mm.

An empty flask with the adapter in place is evacuated to a pressure of 50 mm. or less, and the adapter is closed with a glass rod, as described above. The evacuated flask is immersed in boiling water for 30 minutes (possibly some kinds of rubber may require longer) and thereafter is attached to the suction pump for 3 minutes. The adapter is then ready for analyses. Preparation of two adapters suffices for a day's analyses.

Once prepared to be approximately CO_2 -free, the rubber pieces can be kept so by storing in a container from which the air is evacuated.

Handling of Plasma—As stated in the introduction, plasma rather than serum should be used. For routine analyses we use oxalated plasma and mix it with citrate buffer in the reaction flask immediately after it is separated from the erythrocytes. The rest of the analysis can be completed at leisure, as the citrate-plasma mixture keeps, without change in its carboxyl nitrogen, for as much as 2 months in the cold ($4-6^\circ$).

To 1 cc. of plasma in a reaction vessel are added 2 cc. of citrate buffer solution and 1 drop (0.05 cc.) of caprylic alcohol. The solution remains fluid enough to permit complete extraction of CO_2 by the prescribed technique. More concentrated solutions of protein may when heated form a gel which interferes with extraction of the CO_2 . More dilute solutions, with total volume greater than 3 cc., would require a longer time of reaction with ninhydrin to complete the evolution of CO_2 from amino acids, and would cause a correspondingly greater correction for the CO_2 slowly evolved by the proteins.

Removal of Bicarbonate CO_2 —Removal of bicarbonate CO_2 is accomplished by applying suction to the flask, reducing the pressure below 50 mm., while vigorous rotary shaking is maintained for 2 minutes.

Removal of Heat-Labile "100° CO_2 "—Some plasmas contain traces of un-

identified material which gives off CO_2 for a few minutes when heated at 100° . We shall call CO_2 of this origin " 100°CO_2 ". Its evolution is nearly complete in 2 or 3 minutes active boiling, and is assured by 10 minutes steaming. It is removed by evacuating the air from the flask containing the buffered plasma sample (see "Technique of handling reaction vessel," p. 389), and by heating the evacuated flask for 10 minutes immersed in boiling water. The flask is then transferred for 2 minutes to a bath at $38-40^\circ$. The evolved " 100°CO_2 " is finally removed from the vessel by attaching the latter to a water suction pump for 2 minutes. The 2 minute suction removes all the CO_2 and also cools the contents of the flask as a preliminary to adding ninhydrin.⁵

Several samples may be heated together to remove the " 100°CO_2 ." Only two of the adapters used at this stage need to have been prepared free of rubber CO_2 .

Ninhydrin Reaction—After removal of bicarbonate CO_2 and " 100°CO_2 ," the adapter is disconnected from the flask, and 100 mg. of ninhydrin are measured from a calibrated glass spoon into the flask. An adapter, *previously treated to free it from rubber CO_2* , is attached to the flask, and the flask is immersed in boiling water for exactly 10 minutes. In the reaction with ninhydrin *the timing must be exact* at 10 minutes; otherwise, as shown by Fig. 3, the correction for CO_2 formed by the proteins will not be accurate.

Absorption of CO_2 by Alkali in Van Slyke-Neill Chamber—This operation is carried out as described on pp. 643-646 of Van Slyke, Dillon, MacFadyen, and Hamilton (3). The connection of the reaction vessel and the manometric chamber (Fig. 1, *D*) is made as described on p. 389 under "Technique of handling reaction vessel." Lowering the mercury in the chamber five times suffices to insure complete transfer of the CO_2 , instead of the eight times required in the original method ((3) p. 645), when 3 cc. of solution are in the reaction vessel. The use of salt-saturated alkali in the chamber accelerates the transfer, perhaps because the vapor pressure in the chamber is lowered.

Determination of CO_2 in Manometric Apparatus—This procedure is as described on p. 646 of Van Slyke *et al.* (3), with one exception. The saturation of the solution in the chamber with salt retards the evolution of the CO_2 when the solution is acidified and shaken. Whereas 1 minute's shaking with the mercury at the 50 cc. mark sufficed to obtain equilibrium in the original method, at least 2.5 minutes are required when the salt-saturated reagents are used, and 3 minutes provide a safer margin.

In sealing the cock of the chamber after "ejection of the unabsorbed gases," the bottle shown in Fig. 1, *E* is convenient.

⁵ The necessity of cooling the reaction solution prior to addition of ninhydrin is discussed in detail on p. 640 of the previous paper (3), under the heading "Chilling the solution."

Analyses in Series—In running a number of analyses it is convenient to remove the "100° CO₂" by heating all the flasks and their samples together. Then the heatings with ninhydrin are carried out separately in series; each sample after the first is heated with ninhydrin while the preceding analysis is being put through the CO₂ determination, from "Absorption of CO₂ by alkali in Van Slyke-Neill chamber," to the end of the analysis. By this procedure the CO₂ in each analysis is determined immediately after the reaction with ninhydrin, and the danger of having any measurable part of the evolved CO₂ pass into the rubber stopper is less than if a number of flasks were put through the ninhydrin reaction together and then permitted to stand for varying periods before the CO₂ was determined. As shown previously ((3) foot-note, pp. 632-633) it is during such standing rather than during the heating of the reaction period that CO₂ absorption by the rubber is most likely to occur.

When analyses are run in series as described above, it suffices to have only two adapters freed of rubber CO₂ by preliminary heating *in vacuo*. These are used in alternate analyses.

Calculation of Results with Correction for Protein CO₂ Estimated from Plasma Protein Content—The pressure of CO₂ evolved in the ninhydrin reaction is calculated as

$$P_{CO_2} = p_1 - p_2 - c$$

as in the original method (3), *c* being the value of $p_1 - p_2$ obtained in a blank analysis.

The carboxyl nitrogen content of the plasma in mg. per 100 cc. is calculated as

$$\text{Mg. COOH-N per 100 cc.} = P_{CO_2} \times \text{factor} - (\text{protein CO}_2 + \text{urea CO}_2)$$

(The term *carboxyl nitrogen* and its symbol, *COOH-N*, are used as previously defined ((3) p. 638).) The *factor* is from Table I. The *protein CO₂ + urea CO₂* can be calculated as 0.00077 protein N + 0.0026 urea N, where all values are expressed as mg. of N per 100 cc. of plasma. (In place of 0.00077 *protein N* one may substitute $0.125 \times \text{gm. of protein per 100 cc.}$) The correction is most conveniently computed by the line chart in Fig. 2. As indicated by the line drawn from 10 mg. of urea N to 7 gm. of protein per 100 cc., the correction for protein and urea in a plasma with normal contents of these substances is about 0.9 mg. of carboxyl N per 100 cc., only 0.026 mg. being contributed by the urea. Except in uremic cases, the urea correction is so slight that a urea N content of 15 mg. per 100 cc., and a urea CO₂ correction of 0.04 mg. of COOH-N per 100 cc., can be assumed. The protein content for purposes of the correction can be estimated accu-

rately enough from the specific gravity of the plasma by the formula of Moore and Van Slyke (5); viz.,

$$\text{Gm. protein per 100 cc. plasma} = 343 (\text{sp. gr.} - 1.007)$$

The factors in Table I are computed by Equation 1 of Van Slyke and Folch (6) by using the constants $S = 3$, $A = 50$, $\alpha' = 0.307 \times \alpha'$ for CO_2 in water. For $a =$

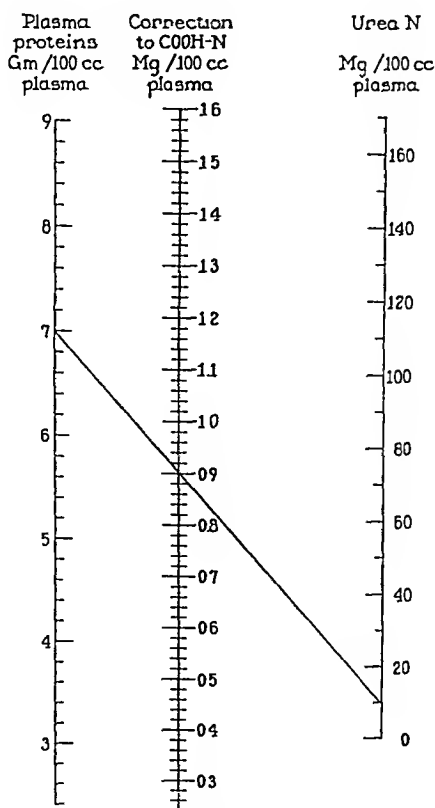


FIG. 2. Line chart for estimating correction due to CO_2 evolved by protein and urea.

0.5, $i = 1.006$; for $a = 2.0$, $i = 1.003$; for $a = 10.0$, $i = 1.001$. The α' of the salt-saturated acid lactate solution from which the CO_2 is extracted and also the i values were determined by the method of Van Slyke (7). The factors for carbon were multiplied by 14.01/12.01 to transform them to factors for nitrogen.

Calculation of Results with Correction for Protein CO_2 Determined by a Second Heating—The evolution of CO_2 from protein proceeds at a constant rate for at least an hour. Hence, after the amino acids are destroyed by

the ninhydrin, if the 10 minutes heating of the reaction mixture is repeated, the amount of CO_2 formed during the reheating will be the same as the amount formed from protein during the 10 minute reaction of the analysis

TABLE I

Factors by Which P_{CO_2} Is Multiplied to Obtain Mg. of Carboxyl Nitrogen

For use when the 0.5 N NaOH and 2.0 N lactic acid employed in the analysis are made up in 25 per cent NaCl solution.

Temperature °C.	Factors		
	$a = 10.0$ $f = 1.001$	$a = 2.000$ $f = 1.003$	$a = 0.500^*$ $f = 1.006$
15	0.008003	0.001603	0.0004020
16	7970	1597	04
17	37	90	3987
18	03	84	70
19	7871	78	54
20	39	71	38
21	07	65	22
22	7776	59	06
23	45	52	3890
24	14	46	75
25	7685	40	61
26	56	34	46
27	27	28	31
28	7598	23	16
29	69	17	02
30	40	11	3787
31	12	05	72
32	7484	1499	58
33	56	94	45
34	27	88	31
35	7300	82	17

* To calculate carboxyl nitrogen directly in mg. per 100 cc. of plasma when the sample analyzed is 1 cc. of plasma, multiply P_{CO_2} by 100 times the factor in the last column; e.g., by 0.03938 when the temperature is 20°.

proper, and can be deducted as the protein correction.* (The CO_2 evolved from urea during the reheating is negligible, as will be shown in the experimental part.)

* The validity of this correction for proteins is discussed on p. 656 (3).

To determine the protein correction for any analysis, after the CO_2 formed from amino acids has been transferred to the Van Slyke-Neill chamber, the reaction vessel is disconnected from the chamber, and during the disconnecting the pinch-cock is applied so that the vacuum in the vessel is maintained. The pinch-cock is then replaced by the glass rod, the vessel is heated another 10 minutes in the steam bath, and the CO_2 formed during this heating is measured.

To distinguish the CO_2 evolved from amino acids + protein + urea in the ninhydrin reaction during the first heating from the protein CO_2 evolved during the second heating (urea CO_2 is negligible in the *second* heating), the subscripts I and II are applied to the pressure readings in the following calculations.

1st heating, $(P_{\text{CO}_2})_I = (p_1 - p_2 - c)_I$; 2nd heating, $(P_{\text{CO}_2})_{II} = (p_1 - p_2 - c)_{II}$

The pressure P_{CO_2} of CO_2 from amino acid carboxyl groups is calculated by subtracting $(P_{\text{CO}_2})_{II}$ from $(P_{\text{CO}_2})_I$.

$$P_{\text{CO}_2} = (p_1 - p_2)_I - (p_1 - p_2)_{II}$$

c cancels out in the subtraction, since it occurs in both $(P_{\text{CO}_2})_I$ and $(P_{\text{CO}_2})_{II}$. The carboxyl nitrogen is then calculated as

$$\text{Mg. carboxyl nitrogen} = P_{\text{CO}_2} \times \text{factor minus } 0.0026 \text{ urea N}$$

The urea correction, 0.0026 urea N, as stated, can be assumed to be equivalent to 0.04 mg. of COOH-N per 100 cc. of plasma, except in cases of pathologically high blood urea.

Examples of Analyses of Normal Plasmas

A. Calculation with protein correction estimated from plasma protein content

$p_1 = 264.0$ mm., $p_2 = 110.1$, $c = 24.0$ mm., temperature = 24.0° , factor = 0.03875
Protein concentration = 7 gm. per 100 cc. Urea N = 10 mg. per 100 cc. $P_{\text{CO}_2} = 264.0 - 110.1 - 24.0 = 129.9$ mm.

COOH-N per 100 cc., uncorrected for protein and urea $\text{CO}_2 = 0.03875 \times 129.9 = 5.00$ mg.

Protein + urea correction from nomogram = 0.90 mg. COOH-N

COOH-N corrected = $5.00 - 0.90 = 4.10$ mg. per 100 cc.

B. Calculation with protein correction estimated from second heating

First heating $p_1 = 264.0$ mm., $p_2 = 110.1$

Second heating $p_1 = 159.1$, $p_2 = 110.5$; temperature = 24.0°

$P_{\text{CO}_2} = (p_1 - p_2)_I - (p_1 - p_2)_{II} = 153.9 - 48.6 = 105.3$

COOH-N corrected = $(105.3 \times 0.03875) - 0.03 = 4.05$ mg. per 100 cc.

EXPERIMENTAL

Quantitative Recovery of Amino Acids Added to Plasma, to Serum, and to Erythrocyte Solution

The results are shown in Table II.

Behavior of Urea under Conditions of the Analysis—As previously pointed out (3), urea heated with water plus ninhydrin forms less CO_2 than if the

heating is done without ninhydrin, the depressing effect of ninhydrin on the CO_2 evolution being evidence that ninhydrin combines with urea to form a compound which evolves CO_2 less readily than does the free urea. This behavior is shown in Table III, by comparing the results under (A) with those under (B). Furthermore, in the *second* heating with ninhydrin, almost no CO_2 was evolved, indicating that during the first heating the com-

TABLE II

Recovery of Amino Acids Added to Plasma, Serum, or Erythrocytes

Protein and urea corrections were made by the nomogram of Fig. 2.

Blood fraction	COOH-N found before addition of amino acids	Amino acids added		COOH-N found after adding amino acids	COOH-N recovered	
		Substance	Amount of COOH-N			
	mg. per 100 cc.		mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	per cent of added COOH-N
Plasma 1	2.91	Arginine monochloride	64.66*	67.96	65.05	100.6
" 2	4.27	" "	4.57*	8.80		
	4.31	" "	4.57	8.82	4.52	99.1
" 3	5.30	Aspartic acid	45.70*	50.98	45.68	98.7
" 4	3.54	Hexone bases from gelatin	5.32†	8.95	5.41	101.5
" 5	4.19	Monoamino acids from gelatin	29.22†	33.27	29.08	99.5
" 6	3.59	Cascin pancreatic digest	31.99†	35.55		
	3.55	" "		35.78		
	3.58	" "		35.33	31.98	100.0
Serum 1	5.69	" "	24.37†	30.29		
	5.85	" "		30.51	24.37	101.1
Erythrocyte solution	3.41	" "	24.37†	27.88	24.47	100.4

* Theoretical from weighed addition of pure amino acid. The amounts of arginine monochloride added to Plasmas 1 and 2 were 980.0 and 34.28, of aspartic acid in Plasma 3, 220.0 mg. per 100 cc. Separate analyses of the preparations gave 99.2 per cent of theoretical carboxyl N for the arginine and 98.7 per cent for the aspartic acid, which is unique in evolving 2 moles of CO_2 (3).

† From analytically determined COOH-N of added amino acid mixture.

bination of urea to form a heat-stable compound with ninhydrin was almost complete.

The results in Table III were obtained by putting urea solutions of the indicated concentrations through the procedures described for plasma analyses, with second heatings as described for obtaining the protein correction. In the experiments under (A), however, the ninhydrin was omitted, in order to show the rate of urea hydrolysis in absence of ninhydrin; and in the analyses under (C) a known amount of arginine was added

in order to show the non-effect of urea on the reaction of ninhydrin with amino acids under the conditions of the plasma analyses, even when urea is present in more than maximal uremic concentration.

Experiments Showing That the "100° CO₂" Evolved by Heat-Labile Substances Other Than Urea and Protein in Plasma Is the Same Whether Ninhydrin Is Present or Absent during Heating—To demonstrate this point the "100° CO₂" was measured by two procedures, in one of which the "100° CO₂" was evolved by heating without ninhydrin, while in the other it was

TABLE III

Behavior of Urea under Conditions of Plasma Analysis

The results show (A) amounts of CO₂ formed by hydrolysis of urea heated at pH 2.5 in absence of ninhydrin; (B) the smaller amounts formed when ninhydrin is present; (C) failure of urea, even in larger proportions than in uremic blood, to prevent quantitative reaction of ninhydrin with an amino acid.

Urea N concentration	(A) No ninhydrin; no amino acid				(B) 100 mg. ninhydrin; no amino acid			(C) 100 mg. ninhydrin; 38.0 mg. amino acid; COOH-N*		
	1st heating		2nd heating		1st heating		2nd heating	1st heating	2nd heating	
	Carboxyl nitrogen equivalent to CO ₂ formed									
	From urea								From urea and amino acid	
	mg. per 100 cc.	mg. per 100 cc.	per cent of urea N	mg. per 100 cc.	per cent of urea N	mg. per 100 cc.	per cent of urea N	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
0	0	0	0	0	0	0	0	37.89	0	
56	0.65	1.16	0.61	1.11	0.17	0.30	—0	38.10	0	
140	1.56	1.11	1.44	1.03	0.35	0.25	—0.03	38.34	0.04	
280	3.20	1.14	3.03	1.08	0.68	0.24	0.05	38.82	0.09	
Mean percentage of urea N		1.14		1.07		0.26				

* 571.4 mg. of arginine monochloride per 100 cc.

evolved in the presence of ninhydrin. In the experiment without ninhydrin, after the bicarbonate CO₂ had been removed at room temperature by evacuation of the acid-buffered mixture, the latter was merely heated 10 minutes in the bath, and the CO₂ formed was determined. In the experiment with ninhydrin two plasma analyses were made, as described for routine analyses, except that in one of them the preliminary heating to remove "100° CO₂" was omitted. In both of these analyses the protein CO₂ was determined, as described for routine analyses, by a second heating of the ninhydrin-plasma mixture. The results are given in Table IV.

It will be noted in column (C) that the correction for protein CO₂ was

higher by 0.18 mg. per 100 cc. when the plasma was preheated to remove "100° CO₂" (bottom line) than when the preheating was omitted (line above). This effect of preheating the protein without ninhydrin, in increasing the rate of protein CO₂ evolution after ninhydrin was added, was noted in other similar experiments. Apparently the heat denaturation of the proteins makes them more labile with respect to this CO₂

TABLE IV

"100° CO₂" Determined Directly by Heating without Ninhydrin and Indirectly As Difference between Ninhydrin Analyses with and without Preheating

Method of determining "100° CO ₂ "	Preheating to remove "100° CO ₂ " before ninhydrin addition		COOH N indicated by CO ₂ evolved in subsequent heatings with ninhydrin		Correction for CO ₂ from urea in Heating I*	COOH N corrected for urea and protein CO ₂	"100° CO ₂ " in equivalents of COOH N
	Pre heated or not	COOH N equivalent to CO ₂ formed = "100° CO ₂ " (A)	Heating I (B)	Heating II (= correction for CO ₂ from protein) (C)			
		mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc	mg per 100 cc
Direct determination of "100° CO ₂ " evolved in preheating without ninhydrin	+	0 67	Heatings with ninhydrin omitted		0 09	0 58 (A - D)	0 58
Difference between COOH-N determinations with and without preheating in advance of ninhydrin reaction	0	0	6 48	1 11	0 02	5 35 (B - C - D)	0 61
	+	Not measured	6 05	1 29	0 02	4 74 (B - C - D)	

* The urea N of the plasma was 8.0 mg per 100 cc. The corrections in column (D) are calculated from the mean percentages, 1.14 and 0.26, at the bottom of Table III, for CO₂ evolved by urea without and with ninhydrin respectively.

Variability of "100° CO₂" in Different Plasmas—The experiments (Table V) were carried out by the same two types of technique by which the results in Table IV were obtained. They show values for "100° CO₂" varying in different plasmas between 0 and 0.6 mg per 100 cc, in equivalents of COOH-N.

Determination of CO₂ Evolved from Plasma Proteins under Conditions of Ninhydrin Reaction—It has previously been shown (3) that proteins heated with ninhydrin under the conditions of the analysis evolve slight amounts of CO₂ at a constant rate for more than an hour. For this reason it is pos-

sible to determine the correction for protein-evolved CO_2 in plasma analyses by first, through 10 minutes heating with ninhydrin, removing all the CO_2 evolved by amino acids, and then carrying through a second heating, during which only the slowly evolved CO_2 from the proteins continues to form. That this slowly evolved CO_2 all arises from the proteins, or at least from substances precipitable with the proteins, is shown by the fact that after deproteinization of plasma Van Slyke and Hamilton (to be published) find that the source of this CO_2 is removed.

TABLE V
Variability of "100° CO_2 " in Different Plasmas

All results are in terms of mg. of COOH-N per 100 cc. of plasma equivalent to the CO_2 formed.

Bicarbonate CO_2 was removed by preliminary evacuation.

Plasma No.	Direct determination of "100° CO_2 " evolved by preheating without ninhydrin			"100° CO_2 " estimated as difference between COOH-N determination with and without preheating		
	COOH-N equivalent to CO_2 formed	Urea N content of plasma*	"100° CO_2 " in COOH-N equivalents†	COOH-N determined without preheating‡	COOH-N determined after preheating‡	"100° CO_2 " in COOH-N equivalents
	(A)	(B)	(C) = (A - 0.0114 B)	(D)	(E)	(F) = (D - E)
	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
I	0.54	9.0	0.44	3.27	2.86	0.41
II	0.14	15.6	-0.04			
III	0.13	10.1	0.02	5.76	5.86	-0.10
IV	0.64	8.1	0.55	5.32	4.73	0.59
V	0.15	11.7	0.02			
VI	0.73	11.7	0.60			
VII	0.44	12.3	0.30			

* Plasma urea N determined by the gasometric urease method of Van Slyke (8).

† The total column (A) is corrected by subtracting 1.14 per cent of the urea N (see Table III).

‡ Each COOH-N analysis was carried out with two successive heatings with ninhydrin, and the corrections for protein and urea were made as in Table IV.

In order to relate the rate of slowly evolved CO_2 formation to the protein content, plasmas of varying protein content were heated 10 minutes with ninhydrin in the usual manner, and the CO_2 formed (amino acid CO_2 and "100° CO_2 ") was removed by evacuation on the water pump. Without releasing the vacuum each flask was returned to the steam bath for 6, 10, or 15 minutes, and the CO_2 evolved during this second heating was measured. The results in mg. of carboxyl nitrogen per 100 cc. equivalent to the CO_2 formed in the second heating are shown in Fig. 3.

Difference between Amino Acid Contents of Serum and Plasma

Serum always contains more measurable COOH-N than plasma. The COOH-N in serum can amount to 110 to 140 per cent of that in plasma. The excess cannot be ascribed to the osmotic action of anticoagulants, be-

Correction for slowly reacting substances (proteins)

Mg. COOH-N per 100 cc. plasma

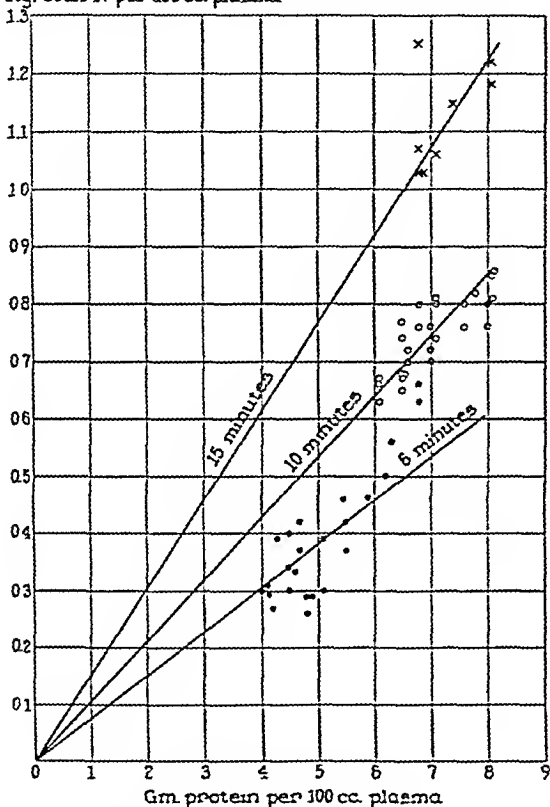


FIG. 3. Relation of CO₂ evolved by plasma proteins to the amount of protein and to duration of the heating period.

cause plasma obtained with the aid of ovalate, of heparin, and without anticoagulant yields the same amount of COOH-N, and this amount is significantly lower than that obtained from serum of the same blood. The

results of an illustrative experiment are shown in Table VI. They were obtained with use of the all-glass reaction vessel of Van Slyke, Dillon, MacFadyen, and Hamilton ((3) Fig. 1, C). In this experiment, the plasma obtained without anticoagulant was allowed to clot, and the serum from it was separated immediately after the clot had formed.

In subsequent experiments, not tabulated, the blood was drawn into chilled, paraffined tubes and the plasma was centrifuged without anticoagulant. The plasma was separated and allowed to clot and the serum was analyzed for COOH-N at different intervals. The results indicated that the COOH-N began to increase within 2 hours, and that after several hours a level of COOH-N was reached which was 40 to 60 per cent higher

TABLE VI

Results Showing (A) Non-effect of Anticoagulant on Carboxyl N of Plasma and (B) Increase of Serum Amino Acid Content during Clotting

All analyses were made on material from the same blood.

	Blood fraction	Carboxyl N
		mg. per 100 cc.
A	Oxalate plasma	4.53
		4.56
	Heparin "	4.44
		4.47
B	Serum from plasma clotted in absence of cells; no anti-coagulant present	4.72
		4.68
		4.65
	Serum from whole blood, clotted in presence of cells	5.27
		5.33

than that at the start of clotting. It follows that serum formed by clotting of plasma either in the presence or the absence of erythrocytes will contain more or less of this extra COOH-N, depending on the clotting time. A more detailed study of clotting and anticoagulants is in progress and will be reported at a later date. In the meantime, the recommended material for analysis is plasma obtained with oxalate, heparin, or citrate as anti-coagulant.

SUMMARY

The gasometric determination of amino acids by their specific reaction with ninhydrin to evolve CO₂, described by Van Slyke, Dillon, MacFadyen, and Hamilton (3), has been applied to blood plasma under conditions which

avoid the necessity of removing the proteins or the urea. These substances evolve small amounts of CO_2 , but corrections for them can be accurately applied.

In manometric analyses by the ninhydrin- CO_2 method, particularly in the submicroanalyses used for plasma, it has been found advantageous to saturate with NaCl the 0.5 N NaOH and 2 N lactic acid introduced into the manometric chamber, in order to diminish the solubility of CO_2 in the final acidified mixture. Factors are given for use with the salt-saturated reagents for general, as well as for plasma, analyses.

When either whole blood or separated plasma clots, the amino acid content of the serum formed is usually 10 to 40 per cent greater than that of the plasma. To avoid error from this artifact plasma rather than serum should be used for the analysis. The mechanism by which amino acids are set free during clotting will be the object of further study.

The present method owes much of its practicability to continuous and congenial collaboration with Dr. Paul Hamilton and Dr. Donald D. Van Slyke.

BIBLIOGRAPHY

- 1 Farr, L E, and MacFadyen, D A, *Proc Soc Exp Biol and Med*, 42, 444 (1939), *Am J Dis Child*, 59, 782 (1940)
- 2 Farr, L E, and Alpert, L K, *Am J Physiol*, 128, 772 (1940) Farr, L E, MacLeod, C. M, Futeher, P H, Emerson, K, Mirick, G S, and Curnen, E C., *Proc Soc Exp Biol and Med*, 44, 290 (1940) Goettsch, E, Lytle, J D., Grim, W M, and Dunbar, P, *J Biol Chem*, 144, 121 (1942)
- 3 Van Slyke, D D, Dillon, R T, MacFadyen, D A, and Hamilton, P, *J Biol Chem*, 141, 627 (1941)
- 4 Van Slyke, D D, and Neill, J M, *J Biol Chem*, 61, 523 (1924) Peters, J P, and Van Slyke, D D, *Quantitative clinical chemistry, Methods*, Baltimore (1932)
- 5 Moore, N. S, and Van Slyke, D D, *J Clin Int*, 8, 337 (1930)
6. Van Slyke, D. D, and Folch, J, *J Biol Chem*, 136, 509 (1940)
7. Van Slyke, D D, *J Biol Chem*, 130, 545 (1939)
- 8 Van Slyke, D D, *J Biol Chem*, 73, 695 (1927)

THE ACTION OF CERTAIN OXIDANTS AND REDUCTANTS UPON THE ACTIVITY OF BOVINE PHOSPHATASE

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Many enzymes, such as urease (9, 19) cathepsin, papain and papain-like enzymes (9, 8), carbonic anhydrase (12), succinic dehydrogenase (11, 16), and glycerol oxidase (3), are activated by reducing agents and inactivated by oxidizing agents. The activity of crystalline urease appears to be a function of oxidation-reduction potential with a maximum activity at $E_h = +150$ millivolts and a decrease in activity in strongly reducing as well as strongly oxidizing solutions (19). A large group of enzymes appears to be relatively insensitive to oxidizing and reducing agents, although with most of these enzymes the effect of extremely active reductants and oxidants has not been determined. Of this group yeast invertase has been studied with these reagents (17) and it was found that while enzyme activity was unaffected by reductants and mild oxidants over the E_h range from -270 to $+600$ millivolts strong oxidants producing potentials greater than $+600$ millivolts irreversibly inactivated the invertase.

With phosphatase the effect of oxidants and reductants is not clear; certain reducing agents, such as activated hydrogen, sulphydryl compounds, cyanide, and ascorbic acid inhibit, while oxidants like ferrieyanide and iodoacetate do not inhibit (12, 5, 20). The effects of sulphydryl compounds are probably unrelated to their reducing properties, since cysteine may activate, have no effect, or inhibit phosphatase, depending on the conditions of the experiment (20, 15). The present investigation was made in an attempt to clarify the relationship of oxidants and reductants to phosphatase activity.

Methods

Phosphatase was prepared from beef lung and kidney according to Albers (1). The final product was a white powder with activity comparable to the phosphatase preparation of Albers and was generally used at a concentration of 0.1 mg. per ml. of digest. In a typical experiment this amount of enzyme liberated 0.08 mg. of P in 1 hour at 37° . The enzyme preparation contained both alkaline and acid phosphatases. Experiments were also performed with milk phosphatase purified by alcohol precipitation and dialysis, and with a highly purified and very active prep-

aration of alkaline phosphatase obtained from intestine by Dr. Gerhardt Schmidt (*cf.* (18)). In all cases the phosphatase activity was expressed as a percentage of the control which contained no oxidizing or reducing agents.

The method used in determining the phosphatase activity is essentially that of Bodansky (4). In each of ten test-tubes was placed 0.5 ml. of phosphatase plus 0.5 ml. of an oxidant or reductant (usually 1×10^{-3} or 1×10^{-5} M). After temperature adaptation at $37^\circ \pm 0.01^\circ$, to each tube were added 4 ml. of 0.5 per cent sodium glycerophosphate (Merck) dissolved in a 0.442 per cent veronal (Merck) buffer at pH 8.5, adjusted to 37° . After 1 hour 1 ml. samples were removed and added to 1 ml. of 10 per cent trichloroacetic acid, then filtered through Whatman filter paper No. 42. The inorganic phosphate was determined according to Fiske and Subbarow (7) by adding 1 ml. of filtrate to 7 ml. of water, 1.6 ml. of ammonium molybdate reagent, plus 0.4 ml. of aminonaphtholsulfonic acid reagent. 15 minutes were allowed for the color to develop and the concentration of inorganic phosphate was measured with a Spekker photoelectric colorimeter (red filter No. 1, maximum absorption at $640 \text{ m}\mu$) which had been standardized with solutions of known phosphate content. At the concentrations employed the presence of oxidants or reductants did not interfere with the determination of phosphorus (*cf.* (21)).

During the course of the digestion samples were taken from the tubes and the oxidation-reduction potential of the solution measured with the platinum electrode connected to a Beckman pH meter adapted for measuring potentials in millivolts. The E_h of digests containing the very strong oxidants was not stable but tended to decrease somewhat with time; the values recorded are those measured a few minutes after the addition of substrate to the enzyme-oxidant solution.

Results

Preliminary studies on phosphate liberation by phosphatase as a function of time indicated that the reaction follows a smooth curvilinear (almost linear) course over a period of 3 hours. In view of this the amount of P liberated in 1 hour was taken as a measure of rate. In preliminary experiments 1×10^{-2} M concentrations of oxidants or reductants in the digest were used. Since marked inactivation of the phosphatase occurred with some salts, while it did not with other salts at the same E_h , it was concluded that some compounds caused inactivation at this concentration owing to a specific toxicity unrelated to E_h . In all subsequent work this specific toxicity was largely avoided by using 1×10^{-3} M or lower concentrations. All the enzyme preparations had high activity in the absence or in the presence of 2×10^{-3} M MgSO_4 , and the effects of oxidants or reductants seem to bear no relationship to its presence. A preliminary survey was

made of the effects of a variety of different oxidizing and reducing agents on phosphatase activity. From this list were then selected for further study eighteen compounds which appeared to exert little or no inhibiting action unrelated to oxidation-reduction potential.

A dilution of the highly purified intestinal alkaline phosphatase was prepared which liberated 0.06 mg. of P per ml. in 1 hour at 37° in the control. The phosphatase activity was then measured with each one of

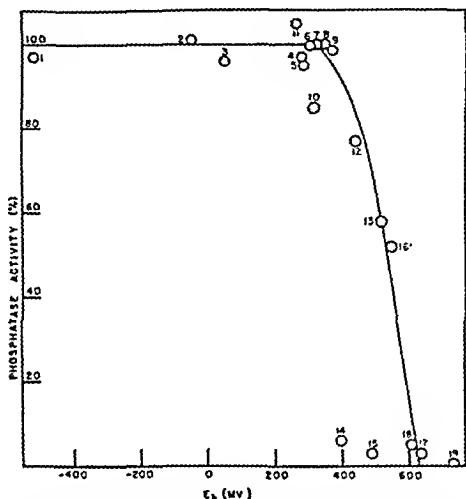


FIG. 1. The activity of intestinal phosphatase expressed as a percentage of the control is plotted against the oxidation-reduction potential of the digest to which has been added one of the following: 1, $H_2 + Pt$ asbestos; 2, 0.1 saturated H_2S ; 3, $1 \times 10^{-3} M Na_2S$; 4, $1 \times 10^{-3} M K_4Fe(CN)_6$; 5, $1 \times 10^{-3} M Na_2SO_3$; 6, $1 \times 10^{-3} M Na_2S_2O_3$; 7, $1 \times 10^{-3} M H_2O_2$; 8, water; 9, $1 \times 10^{-3} M$ sodium iodoacetate; 10, $1 \times 10^{-3} M KCNS$; 11, $1 \times 10^{-3} M$ sodium methionate; 12, $1 \times 10^{-3} M K_2Cr_2O_7$; 13, $1 \times 10^{-3} M K_4Fe(CN)_6$; 14, $1 \times 10^{-3} M CrCl_3$; 15, $1 \times 10^{-3} M Br_2$; 16, $1 \times 10^{-3} M I_2-KI$; 17, $1 \times 10^{-3} M I_2-KI$; 18, $1 \times 10^{-3} M KMnO_4$; 19, $1 \times 10^{-3} M KMnO_4$.

eighteen different oxidants or reductants, and the enzyme activity expressed as a percentage of the control. From Fig. 1, where phosphatase activity is plotted against the E_h of the digest, it is apparent that the points for the various agents are not scattered at random but fall roughly along a curve. The activity of intestinal phosphatase seems to be independent of the oxidation-reduction potential over the range from -500 to $+350$ millivolts. Above the latter potential the activity falls rapidly, reaching zero at about $+650$ millivolts. The similarity of these results to those

reported for invertase (17) are striking and suggest a common mechanism of enzyme inactivation by strong oxidants. The experiment was repeated several times with intestinal phosphatase subsequently purified by $\text{Al}(\text{OH})_3$ adsorption, with very similar results.

Essentially comparable results were obtained in a large number of experiments with phosphatase prepared from other bovine tissues. In *A* and *B* of Fig. 2 are shown the results with lung and kidney phosphatases prepared according to Albers (1), and in *C*, the results with milk phosphatase purified by dialyzing skim milk in Ceneo dialyzer tubing. The

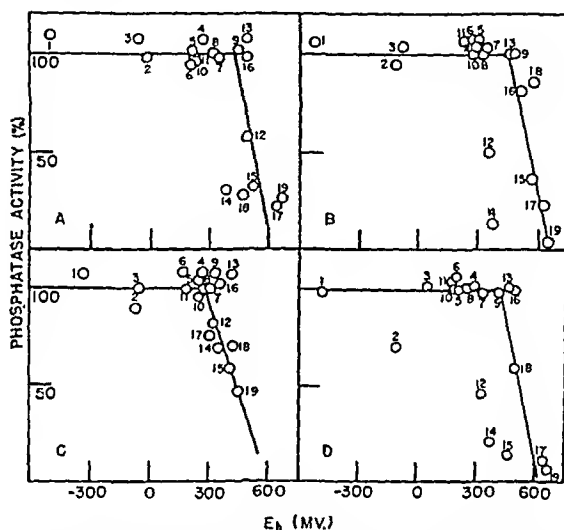


FIG. 2. Per cent phosphatase activity is plotted against the oxidation-reduction potential of the digest. The numbers refer to the same oxidants or reductants as in Fig. 1. *A*, lung phosphatase; *B*, kidney phosphatase; *C*, milk phosphatase; *D*, kidney phosphatase (acid).

lung phosphatase seems to be stable up to slightly higher potentials than are the other preparations. The milk phosphatase preparation contains reducing systems (indicated by the rapid rate of decolorization of permanganate) as impurities which react with the strong oxidants and prevent them from completely inactivating the enzyme. Although several different methods were used to separate the phosphatase from these reducing systems, the results were only partially successful. Despite the scatter of the plotted data, the similarity of results with phosphatase preparations from different tissues is striking; for all the preparations used (including phosphatase from human duodenal juice¹) the enzyme activity was un-

¹ The author is indebted to Dr. Harry Shwachman for this sample.

affected by certain reductants and weak oxidants, but was greatly decreased by strong oxidants. In general, the effects of these compounds on phosphatase were unrelated to its degree of purity with the possible exception that the most highly purified preparations may be somewhat less stable in the presence of strong oxidants. Dialysis of several different phosphatase preparations, which removed the Mg^{++} and other impurities, did not modify the usual effects of oxidants and reductants upon the enzyme activity.

Action of Phosphatase on Other Substrates—If oxidizing and reducing compounds act primarily upon the enzyme rather than the substrate, then their effects should be relatively independent of the particular substrate employed. With the same technique as was previously used but with calcium hexose diphosphate, disodium phenyl phosphate, or $Na_4P_2O_7$ in place of the sodium glycerophosphate, the effects of oxidants and reductants on the activity of kidney phosphatase were studied in the usual manner. Results with all three substrates were similar to those with glycerophosphate, indicating inactivation of the phosphatase by strong oxidants but no effect of the other agents employed.

Acid Phosphatase—While it appears that acid and alkaline phosphatase are distinct enzymes (13), they usually occur together and have somewhat similar properties except for pH effects. It was therefore of interest to test the action of oxidants and reductants upon the activity of acid phosphatase by adjusting the pH of the digest to 4.5 by use of a veronal-acetic acid buffer. It is apparent from a comparison of Fig. 2, D with the other curves that the results with acid phosphatase are very similar to those with alkaline phosphatase. The point for H_2S is somewhat low, however, possibly because of a slightly toxic action of this reductant at low pH.

Effects of Other Oxidants and Reductants on Phosphatase—A large number of different compounds were tested on lung phosphatase. While most of these affected the enzyme activity in a manner to be expected from their oxidation-reduction potentials (Table I), a large number of reductants, chiefly sulphydryl compounds and cyanide, were found to inhibit phosphatase, in confirmation of the work of others (12, 5, 15, 14). Williams and Watson (20) have suggested that the inhibition of phosphatase by sulphydryl compounds is specific and unrelated to their reducing properties, since in low concentrations cysteine actually produces activation and since the inhibition by higher concentrations is reversed by iodoacetate just as is the inhibition by glycine. Similarly, from the present study it appears that the inhibition produced by such reductants is not related to their reducing power *per se*, but is due to a specific toxicity not shared by other reductants.

Reversibility of Phosphatase Inactivation by Oxidants—The question arises whether oxidants reversibly inactivate phosphatase as with urease (19)

TABLE I

Activity of Lung Phosphatase As Affected by 1×10^{-3} M Oxidants and Reductants

Salt	E_h of digest	Phosphatase activity	Salt	E_h of digest	Phosphatase activity
	mv.	per cent		mv.	per cent
Cysteine.....	+70	35	Na_2HAsO_3	+246	100
Cystine*.....	+366	77	Na_3AsO_4	+426	100
Thioglycolic acid....	+134	59	FeCl_2	+426	80
Glutathione.....	+206	68	$\text{Fe}(\text{NO}_3)_3$	+536	10
KCN.....	+296	81	KIO_3	+546	100
NH_2OH	+76	76	KClO_4	+521	16
SnCl_2	+151	13	KBrO_3	+475	86
$\text{Na}_2\text{S}_2\text{O}_4$	+116	34	NaBrO_3	+424	89
$\text{K}_2\text{S}_2\text{O}_8$	+420	84	$\text{NH}_4\text{H}_2\text{PO}_2$	+366	106
$\text{Na}_2\text{S}_2\text{O}_6$	+486	65	Quinone*.....	+609	16
NaSeO_3	+366	97	Quinhydrone*.....	+369	59
Na_2SeO_4	+516	101	Benzoyl peroxide*....	+463	76

* 0.1 saturated solution.

TABLE II

Reactivation of Phosphatase by Dialysis or by Na_2S after Inactivation by Strong Oxidants

Oxidant	Treatment	E_h of digest	Phosphatase activity
		mv.	per cent
1×10^{-3} M KMnO_4	None	+666	2
1×10^{-3} " ".....	Dialysis	476	2
1×10^{-3} " ".....	2×10^{-3} M Na_2S	296	1
1×10^{-4} " ".....	None	476	12
1×10^{-4} " ".....	Dialysis	426	33
1×10^{-4} " ".....	6×10^{-5} M Na_2S	276	35
1×10^{-4} " ".....	1.2×10^{-4} M Na_2S	246	78
1×10^{-5} " ".....	None	426	74
1×10^{-5} " ".....	Dialysis	386	91
1×10^{-3} " I_2 -KI.....	None	636	4
1×10^{-3} " ".....	Dialysis	400	1
1×10^{-3} " ".....	1.2×10^{-3} M Na_2S	436	9
1×10^{-3} " ".....	2×10^{-3} M Na_2S	316	31
1×10^{-4} " ".....	None	516	19
1×10^{-4} " ".....	Dialysis	376	54
1×10^{-4} " ".....	6×10^{-5} M Na_2S	306	81
1×10^{-4} " ".....	2×10^{-4} " "	306	95
1×10^{-3} " Br_2	None	446	0
1×10^{-3} " ".....	Dialysis	368	31
1×10^{-3} " CrCl_3	None	446	12
1×10^{-3} " ".....	Dialysis	364	101
1×10^{-3} " $\text{K}_2\text{Cr}_2\text{O}_7$	None	426	48
1×10^{-3} " ".....	Dialysis	358	64
0.1 saturated quinone.....	None	609	8
0.1 " ".....	Dialysis	356	58

or do so irreversibly as with invertase (17). This problem can be studied by inactivating phosphatase with an oxidant, then subsequently removing the oxidant by dialysis. The results of a typical experiment are shown in Table II from which it is apparent that the inactivation of phosphatase by strong oxidants is partially or completely reversible on dialysis for 100 minutes, except for $1 \times 10^{-3} \text{ M}$ KMnO_4 and $\text{I}_2\text{-KI}$ for which inactivation is irreversible.

The problem of the reversibility of inactivation can also be investigated by adding a reducing agent to the enzyme after it has been inactivated by an oxidant, then measuring any change in phosphatase activity. It is apparent from Table II that, as before, $1 \times 10^{-3} \text{ M}$ solution of KMnO_4 produces irreversible inactivation, while inactivation produced by $1 \times 10^{-4} \text{ M}$ KMnO_4 , $1 \times 10^{-3} \text{ M}$ $\text{I}_2\text{-KI}$, or $1 \times 10^{-4} \text{ M}$ $\text{I}_2\text{-KI}$ is reversed by adding Na_2S . The extent of the reactivation obtained with Na_2S is greater when the amount of this reductant added to the system is increased. A comparison of the two different methods of reactivation reveals that while phosphatase inactivated by $1 \times 10^{-3} \text{ M}$ $\text{I}_2\text{-KI}$ cannot be reactivated by dialysis the addition of Na_2S can produce at least partial reactivation.

Mechanism of Phosphatase Inactivation by Strong Oxidants—Oxidation of substituent sulfhydryl groups in the phosphatase molecule, while it may be an adequate explanation of inactivation of enzymes like urease (9, 19), cannot account for the inactivation of invertase (17) and phosphatase for which stronger oxidants than ferricyanide and iodoacetate, which oxidize sulfhydryl groups (2), are required to inactivate the enzyme. Denaturation of the protein enzyme by strong oxidants is an unlikely explanation of the inactivation of phosphatase in view of the ease with which it can be reactivated by lowering the oxidation-reduction potential. Strong oxidants might react with constituent amino acids of the enzyme such as tyrosine, tryptophane, or histidine and in this way inactivate the enzyme. This is analogous to pepsin inactivation by the iodination of tyrosine in the protein molecule (10). If the latter explanation be true, one would expect a shift in the ultraviolet absorption spectrum of phosphatase upon the addition of strong oxidants in view of the fact that the absorption spectrum of phosphatase is due chiefly to the presence of tyrosine or tryptophane or of both (18). Such a shift would not be expected if oxidants merely denature the protein (6).

Ultraviolet absorption spectra of intestinal phosphatase were taken with a Hilger quartz spectrograph equipped with a Spekker photometer, with a tungsten spark as the light source. The photographic plates were projected and the match points at different wave-lengths determined by eye. The enzyme was dissolved in glass-distilled water and placed in a 2 cm. quartz cell. The duplicate cell in all cases contained only water plus the

oxidant or reductant. The pH of the various unbuffered solutions, including those containing oxidant or reductant, varied between 5.0 and 6.0 as measured with the glass electrode. The concentration of oxidant or reductant in both the enzyme solution and in the duplicate cell was only 1×10^{-6} M, since at higher concentrations the absorption due to the salt interfered with the determinations.

From the results presented in Table III it can be seen that the addition of oxidants but not of reductants to a highly purified preparation of alkaline phosphatase brings about a shift in the absorption maxima and minima toward higher wave-lengths. Results of a similar nature were also obtained with kidney phosphatase. While these data suggest that strong

TABLE III

Ultraviolet Absorption Maxima and Minima of Intestinal Phosphatase, Tyrosine, and Tryptophane

Substance	Oxidant or reductant added, 1×10^{-6} M	Absorption maximum	Absorption minimum
		$m\mu$	$m\mu$
Phosphatase .	None	277	253
"	KMnO ₄	282	257
"	I ₂ -KI	280	253
"	Br ₂	280	254
"	K ₂ Cr ₂ O ₇	280	255
"	Na ₂ S	275	253
Tryptophane	None	280	242
"	KMnO ₄	280	242
Tyrosine	None	276	245
"	KMnO ₄	281	247
Diiodotyrosine*	None	287	258

* Data from Ellinger (6).

oxidants effect an inactivation of phosphatase by oxidizing constituent amino acids of the enzyme such as tyrosine and tryptophane, the argument would be more convincing if the same concentration of oxidants produced a similar shift in the absorption spectrum of the individual amino acids. A study of tyrosine and tryptophane (Table III) indicated that the absorption spectrum of only the former was shifted by KMnO₄. According to Ellinger (6) treatment of tyrosine with iodine to form diiodotyrosine produces a similar shift. From this study it appears likely that strong oxidants inactivate phosphatase by oxidizing certain constituent amino acids such as tyrosine but not tryptophane. For both pepsin (10) and phosphatase the enzyme becomes inactivated when tyrosine is oxidized in the molecule.

I am very grateful to Professor John R. Loofbourow, Dr. Gerhardt Schmidt, and Mr. Herbert Jaffe for valuable assistance in this investigation.

SUMMARY

The activity of bovine alkaline phosphatase is unaffected by most reductants and mild oxidants, but is greatly decreased by strong oxidants. The activity becomes progressively less as the oxidation-reduction potential of the digest is raised above $E_h = +400$ millivolts and reaches zero at $E_h = +650$ millivolts. The results are independent of the tissue from which the enzyme is obtained and independent of the particular substrate employed. Similar results are obtained with acid phosphatase. The inactivation of the enzyme by strong oxidants is reversible, since the activity can be restored at least in part by adding a reductant or by dialyzing away the oxidant.

It is suggested that the inactivation of phosphatase by strong oxidants can be explained by the oxidation of substituent amino acids in the enzyme rather than by oxidation of sulfhydryl groups or by protein denaturation. Tyrosine but not tryptophane is among the amino acids oxidized in phosphatase. This is indicated by the fact that the ultraviolet absorption spectrum of phosphatase, attributable to the tryptophane and tyrosine present, shifts to higher wave-lengths, just as does the absorption spectrum of tyrosine, but not of tryptophane, upon the addition of strong oxidants.

BIBLIOGRAPHY

1. Albers, D., *Z. physiol. Chem.*, **265**, 129 (1940).
2. Anson, M. L., *J. Gen. Physiol.*, **23**, 247 (1940-41).
3. Barron, E. S. G., *Bol. soc. quim. Peru*, **6**, 7 (1940).
4. Bodansky, A., *J. Biol. Chem.*, **101**, 93 (1933).
5. Cloetens, R., *Naturwissenschaften*, **28**, 252 (1940).
6. Ellinger, F., *Absorptions spektroskopie im ultraviolett*, The Hague (1927).
7. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
8. Greenberg, D. M., and Winnick, T., *J. Biol. Chem.*, **135**, 761 (1940).
9. Hellerman, L., *Physiol. Rev.*, **17**, 454 (1937); Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 165 (1939).
10. Herriott, R. M., *J. Gen. Physiol.*, **20**, 335 (1937).
11. Hopkins, F. G., Morgan, E. J., and Lutwak-Mann, B., *Biochem. J.*, **32**, 611, 1829 (1938).
12. Kiese, M., and Hastings, A. B., *Science*, **88**, 242 (1938); *J. Biol. Chem.*, **132**, 281 (1940).
13. Perlmann, G. E., and Ferry, R. M., *J. Biol. Chem.*, **142**, 513 (1942).
14. Pett, L. B., and Wynne, A. M., *Biochem. J.*, **38**, 365 (1934).
15. Pyle, J. J., Fisher, J. H., and Clark, R. H., *J. Biol. Chem.*, **119**, 283 (1937).
16. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).

17. Sizer, I. W., *J. Gen. Physiol.*, **25**, 399 (1942).
18. Sizer, I. W., *Proc. Soc. Exp. Biol. and Med.*, **49**, 700 (1942).
19. Sizer, I. W., and Tytell, A. E., *J. Biol. Chem.*, **138**, 631 (1941).
20. Williams, H. L., and Watson, E. M., *J. Biol. Chem.*, **135**, 337 (1940).
21. Woods, J. T., and Mellon, M. G., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 760 (1941).

THE FORMATION OF EXTRACELLULAR *d*(-)-GLUTAMIC ACID POLYPEPTIDE BY *BACILLUS SUBTILIS*

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The major difficulties encountered in any attempt to study the mechanism of physiological synthesis of proteins are (1) the heterogeneity of substrates in the form of the various amino acids and often other substances required for the production of any ordinary protein, and (2) the fact that such syntheses are apparently obligate intracellular processes and, consequently, are not readily available for the detailed study that is possible on systems susceptible to extracellular manipulation. The second of these considerations is probably in part a consequence of the first, since it is likely that only in the cell does there exist the proper concentration and distribution of amino acids. A further important reason for this apparently obligate intracellularity lies in the fact that the fundamental reaction in the formation of proteins, namely the synthesis of the peptide link between two amino acids, is endothermic, and therefore, as pointed out by Borsook and Huffman (1), can probably occur under physiological conditions only if linked with some energy-yielding reaction. The difficulty of preparing cell-free extracts in which linked reactions will take place or even of identifying those which occur within the cell increases the experimental complications of the problem. If, in addition, the identity of the linked reaction is dictated not only by the energy requirements of the formation of the peptide bond, as such, but also by specific requirements imposed on each bond by the influence of the varying character of the side chains of the amino acids, then the problem becomes extremely complex. In any case, knowledge of the mechanism of the energy-yielding reaction for the formation of the peptide bond between any two amino acids will constitute a step toward the solution of this problem. Experimentation in this direction would be greatly facilitated if a system free of the complications discussed above could be found. Studies on a system possessing promising characteristics for this purpose are presented in this report.

Ivánovics and his collaborators (2, 3) found one component of the capsule of the anthrax bacillus and of many strains of the *mesentericus* group of bacilli to be a polypeptide composed largely of *d*(-)-glutamic acid. They showed, further, that certain of these strains produced this polypeptide in a form soluble in the medium and entirely dissociated from the bacterial cell.

If this peptide consisted solely of glutamic acid, at least one of the complications discussed above would be avoided. Furthermore, the finding by Hehre and Sugg (4) that certain microorganisms are capable of extracellular synthesis of serologically active polysaccharide suggested that some strains of this group might provide an extracellular synthesis of the polypeptide. If this were the case, it would solve the second major difficulty.

Before an attempt was made to secure a cell-free system capable of peptide synthesis, it seemed desirable first to establish quantitatively the composition of the peptide, and then to determine as nearly as possible the precursor substrates utilized in its formation. Although the work of Schoenheimer and his associates (5), and more recently that of Ussing (6), demonstrating the incorporation of amino acids into proteins, would make it seem highly probable that the intact glutamic acid molecule is used as a substrate for the peptide synthesis, it was thought advisable to confirm this probability, because the use of any substrates too far removed in the metabolic chain from the immediate peptide precursors would seriously complicate any attempts to prepare an active extracellular system.

With these objectives in mind, the following studies have been carried out on a strain of *Bacillus subtilis* that was found to secrete into the medium a soluble peptide. It has been demonstrated that this peptide is composed solely of *d*(-)-glutamic acid, and that, in the synthesis of this peptide, the microorganism uses the glutamic acid molecule skeleton as a substrate, but not necessarily as the only substrate.

Microorganism and Medium—Various strains of the *subtilis-mesentericus* group were obtained from the United States Department of Agriculture, Bureau of Plant Industry. Of these a number were selected, the cultural and morphological characteristics of which corresponded most closely to those classified as Group I by Ivánovics (7), who stated that one of the characteristics of this group is the ability to secrete peptide into the culture medium. *Bacillus subtilis*, Strain 41259, received from the Department of Agriculture as No. 712, was chosen as the most favorable with respect to quantity and constancy of production of peptide when grown in a simple medium, and with respect to the relatively slight production of other interfering substances. The choice of culture media was determined by the desirability of using as simple a medium as possible. Fortunately this species is generally non-fastidious in its growth requirements, and this particular strain was found to produce peptide on Sauton's medium, which is made as follows: A tap water solution containing 4 gm. of glutamic acid, 2 gm. of citric acid, 0.5 gm. of K_2HPO_4 , 0.05 gm. of ferric ammonium citrate, 5 ml. of a 10 per cent solution of magnesium sulfate, and 20 gm. of glycerol is adjusted to pH 7.4 with strong ammonia. The whole is diluted to 1 liter with tap water, dispensed into a 3 liter Erlenmeyer flask, and sterilized by

autoclaving at 121° for 15 minutes. (If the magnesium sulfate is added as a dry salt, a precipitate is apt to form.)

The inoculum, a few ml. of a 15 to 18 hour culture of Strain 41259 on Sauton's medium, was washed twice with sterile distilled water and suspended in saline before using. Peptide production in a given time was independent of the maintenance of the culture; inocula of microorganisms grown in a rich medium, such as broth or glycerol-potato-agar, produced no more peptide than those grown on Sauton's medium. When incubated at 34°, growth was rapid and a pellicle formed in 2 or 3 days. Prevention of pellicle formation by shaking each day retarded peptide production.

Determination of Peptide—The amount of peptide in solution can be determined as follows: An aliquot of 30 to 35 ml. of the culture is filtered through a Seitz clarifying filter and, after adjustment to pH 6.0, 5 per cent by volume of a saturated solution of copper sulfate is added to the filtrate. The copper salt of the peptide is highly insoluble at pH 6.0 but dissolves in more acid solutions. If the filtrate is too alkaline, copper glutamate is apt to precipitate. (The filtrates of Strain 41259 were usually approximately neutral in reaction. Other strains were often fairly acid or alkaline.) The copper salt of the peptide soon separates as a fine light green precipitate which rapidly flocculates and then coagulates on standing a short time. After being centrifuged and washed twice with dilute copper sulfate solution, the copper precipitate is suspended in 5 to 10 ml. of water and the solution made 1.5 N with strong HCl. The greater part of the precipitate dissolves, leaving a small amount of reddish brown insoluble material, which is separated by centrifugation and discarded.

The clear light blue supernatant represents a preparation of the peptide that is remarkably free of other nitrogenous substances, since it contains no free glutamic acid, and the glutamic acid liberated by hydrolysis accounts for 100 per cent of the total nitrogen (Table I). A Kjeldahl nitrogen or a glutamic acid determination on this solution corresponds, therefore, to its peptide content. The glutamic acid was determined manometrically by Cohen's method (8) after the solution was concentrated to 2 ml. by evaporation in a graduated centrifuge tube and subjected to a 3 to 4 hour period of hydrolysis with 20 per cent sulfuric acid. Omission of the second ether extraction used in this method saves much time and introduces no error in the results obtained with this material.

Production of Peptide—The rate of production of peptide and other copper-precipitable nitrogenous material is shown in Table II. Under these conditions of growth, the amount of peptide formed reached a maximum on approximately the 7th day and then rapidly decreased, at which time there was a relative increase in the amount of copper-precipitable nitrogenous material other than glutamic acid peptide. The disappearance of the pep-

tide from the medium could be delayed by addition of larger amounts of glycerol to the medium or, to a lesser extent, by increasing the glutamic acid. Greater concentrations of the other constituents of the medium did not produce a similar effect (Fig. 1). The disappearance of peptide in

TABLE I
Analytical Values for Peptide Preparations

Preparation I was a copper precipitate dissolved in 1.5 N HCl and dialyzed against citrate buffer; Preparations II and III, copper precipitate dissolved in 1.5 N HCl.

	Total N	Amino N before hydrolysis	Glutamic acid N after hydrolysis	N ¹⁵
	mg. per ml.	mg. per ml.	mg. per ml.	atom per cent
Preparation I	0.62	0.02	0.60	
“ II	0.478	0.019	0.45	
“ III	0.351		0.32	3.99*
	per cent			
Glutamic acid HCl from Preparation III	7.66			4.00

* The preparation was dialyzed against citrate solution and distilled water previous to N¹⁵ determination and isolation of glutamic acid.

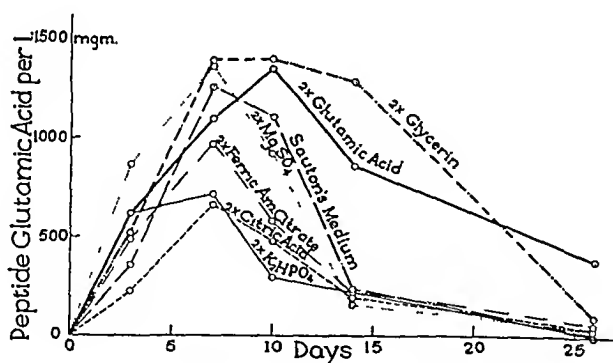


FIG. 1. Effect of increased concentrations of various medium constituents on peptide production.

older cultures is probably due to a hydrolytic enzyme, which will be the subject of further studies.

Preparation of Peptide—For preparative purposes, peptide solutions can be freed of copper by H₂S, but there is usually considerable loss of material, probably due to adsorption by the CuS. On dialysis against distilled water, the acid is removed more rapidly than the copper, which remains in

the sac and precipitates the peptide as the pH rises. Prolonged dialysis against acid presents the possibility of hydrolysis. Dialysis against citrate buffer (0.5 M, pH 5.0) has been found to remove the copper without precipitating the peptide, and the citrate can be removed by subsequent dialysis against distilled water. As would be expected because of the highly acidic character of this peptide, the solutions obtained by this procedure are apt to contain a considerable amount of ash, which can be removed by a 24 hour dialysis against 0.1 N HCl.

Composition and Structure of Peptide—Analytical values for some peptide preparations are shown in Table I. The greatest discrepancies between the total nitrogen and glutamic acid nitrogen values do not exceed 9 per cent, which is within the limits of error of the analytical method. More conclusive proof of the purity of these preparations is furnished by the nitrogen isotope analyses on Preparation III and the purified glutamic acid

TABLE II
Production of Copper-Precipitable Material on Sauton's Medium

Days	Experiment I		Experiment II	
	Glutamic acid N	Total N	Glutamic acid N	Total N
	<i>mg. per l.</i>	<i>mg. per l.</i>	<i>mg. per l.</i>	<i>mg. per l.</i>
4	21.2	22.4		
7	41.2	52.8	50.3	69.6
11	38.6	90.0	33.8	82.4
14	29.1	76.8	14.8	72.0

isolated from it. As will be described later, the experimental conditions leading to the production of Preparation III were such that it was extremely unlikely that other proteins and nitrogenous substances produced by the microorganisms would have the same isotope content as that found for the peptide. Therefore the identity of the isotope content of Preparation III and of the purified glutamic acid isolated from it precludes the presence of other nitrogenous products in this preparation. From these data it can be seen that the peptide consists solely of glutamic acid and therefore meets one of the requirements discussed above.

The titration of a purified peptide solution, with a glass electrode to obtain the end-point, reveals one free carboxyl group per nitrogen atom, as would be expected in a peptide of this composition. A peptide solution containing 0.511 mg. of N required 11.87 ml. of 0.030 N NaOH for neutralization, giving a ratio of N to carboxyl of 1.02.

With the empirical composition of the peptide established, the further problem of its chemical structure presented itself. As pointed out by

Ivánovics and his associates (3), two isomeric forms are possible, depending upon which carboxyl group of the glutamic acid is involved in the peptide linkage. A study of the effect of alkali on the optical activity of peptide solutions offers a convenient approach to this problem. According to the work of Dakin (9), and the elaboration of this work by Levene and Bass (10) and Bovarnick and Clarke (11), if the peptide link is formed by the α -carboxyl group, all except the terminal amino acids of the chain should racemize in alkaline solutions, unless the chain is hydrolyzed into fragments before racemization can take place. If the peptide link is formed by the ω -carboxyl, leaving a free carboxyl group adjacent to the asymmetric carbon atom, racemization should not occur.

A solution of 556 mg. of peptide in 20 ml. of approximately 1 N HCl, $\alpha_D = +0.50^\circ$, was neutralized with NaOH and, after addition of 1.6 ml. of saturated NaOH, the whole was diluted to 25 ml. The rotation of this solution immediately after its preparation was -0.16° in a 2 dm. tube and this value was unchanged after the solution had stood for 10 days at room temperature. During this time, the amino nitrogen increased from 10 to 20 per cent of the total nitrogen, indicating that little hydrolysis had occurred. After another similar experiment, in which there was also no change in rotation, and much less hydrolysis, the peptide was hydrolyzed with strong HCl, and 54 per cent of the total glutamic acid was isolated as the hydrochloride, $[\alpha]_D^{20} = -22.7^\circ$. The combined mother liquors were also levorotatory. These results, in conjunction with a negative biuret test (3), which we have confirmed, strongly indicate peptide linkage involving the ω -carboxyl group.

Substrate for Peptide Formation—After it was found that the peptide consisted solely of glutamic acid, the following experiments were undertaken to determine whether or not the intact glutamic acid molecule was used in the synthesis of the peptide.

1 liter of Sauton's medium was prepared containing 3.0 gm. of *dl*-glutamic acid that had been synthesized as described by Schoenheimer and Ratner (12) by the catalytic reduction of α -ketoglutaric acid in the presence of ammonia and deuterium gas, with palladium black as a catalyst. The deuterium content of this glutamic acid was found to be 12.9 atom per cent excess. The method of Keston, Rittenberg, and Schoenheimer (13) was used for determination of deuterium.

The ammonia in this medium contained isotopic nitrogen, N^{15} , to the extent of 12.23 atom per cent. This was added as ammonia obtained by distillation from isotopic ammonium nitrate (Eastman, $N^{15}H_4NO_3$) in a Kjeldahl apparatus. The receiving flask contained the citric and glutamic acids that were to be incorporated into the medium and, after the distillation, the other ingredients were added. The small washed inoculum used constituted only a negligible nitrogen dilution.

After 4 days incubation the culture filtrate contained 0.311 mg. of peptide glutamic acid per ml., and after 6 days, 0.435 mg. per ml. Growth was interrupted on the 7th day and the entire filtrate was treated with copper sulfate solution. The copper precipitate was triturated with 1.5 N HCl and the insoluble portion discarded. The soluble portion, Preparation II, was refluxed for 4 hours with 15 per cent HCl. After removal of the copper with H_2S , the glutamic acid was isolated as the hydrochloride and recrystallized twice from concentrated HCl.

Analysis—N found, 7.74; theory, 7.63

The N^{15} content of this glutamic acid was 8.1 atom per cent. The N^{15} content of the ammonia present in the culture medium after growth was 11.0 atom per cent.⁶ The average value between this figure and 12.2, the N^{15} content of the ammonia before growth, is 11.6 atom per cent, and it can be assumed that the N^{15} content of any glutamic acid synthesized by the bacteria will closely approximate this value. On this basis the fact that the isolated peptide glutamic acid had an N^{15} value of 8.1 atom per cent indicates that 30.2 per cent of the peptide glutamic acid must have been provided by the glutamic acid in the medium without any rupture of the carbon-nitrogen bond.

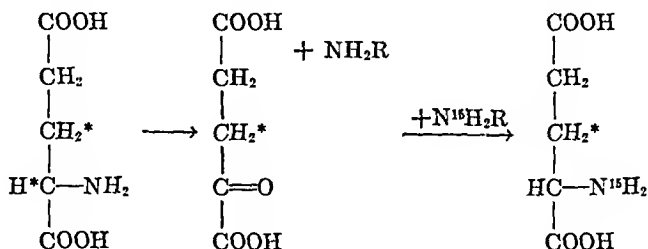
The deuterium content of this glutamic acid hydrochloride was found to be 3.25 atom per cent, or 3.61 atom per cent in terms of glutamic acid. In order to determine the distribution of this deuterium between the α and β positions, the method used by Rittenberg, Ratner, and Hoberman (14) was employed. A 28.1 mg. sample of the hydrochloride (0.153 mm) was diluted with 1984.6 mg. of non-isotopic free glutamic acid (13.49 mm) and then converted to succinic acid by oxidation with chloramine-T. The succinic acid was purified as the barium salt.

Analysis—Ba found, 54.12; calculated, 54.2

The deuterium content of this salt, representing that in the β position of the peptide glutamic acid, was 0.093 atom per cent, which is the equivalent of 3.68 atom per cent when calculated on the basis of glutamic acid. Since the total deuterium of the peptide glutamic acid was found to be 3.61 atom per cent, it is obvious that 100 per cent of the deuterium in the peptide is in the β position in the glutamic acid. By a similar degradation it was determined that the glutamic acid put into the medium contained 9.73 atom per cent in the β position. According to these values for the β -deuterium of the original and peptide glutamic acids, 37.5 per cent of the peptide glutamic acid was derived from that in the medium.

The discrepancy between this value (37.5 per cent) and that arrived at on the basis of the nitrogen isotope analysis (30.2 per cent) is probably due to

the incorporation into the peptide of some of the glutamic acid resynthesized by the bacteria from α -ketoglutaric acid in the reversible reaction



The asterisk designates positions containing deuterium.

involving either the transamination reaction or oxidation with formation of ammonia.

More noteworthy is the fact that, although there is no rupture of the carbon-nitrogen bond in at least 30 per cent of the peptide glutamic acid units, there is, nevertheless, a complete loss of α -deuterium. Since both *d*- and *l*-glutamic acid containing deuterium in the α position were available in the medium, the loss of α -deuterium could not have been merely the result of reactions leading to optical inversion, as the use of either intact isomer would have carried some deuterium into the peptide. The complete absence of α -deuterium, in conjunction with the presence of intact carbon-nitrogen linkages, indicates that α -iminoglutaric acid, $\text{COOHCH}_2\text{CH}_2\text{-NH}$

$\begin{array}{c} \text{NH} \\ || \\ -\text{C}-\text{COOH} \end{array}$, or a derivative thereof, was at some point directly involved in the formation of the peptide bond.

This suggests the possibility that the imino derivative may act as the oxidant in the reaction mechanism in a manner similar to that discussed by du Vigneaud *et al.* (15) who obtained similar results in a study of the *in vivo* acetylation of *d*- α -aminophenylbutyric acid. They postulated a condensation between α -iminobutyric and pyruvic acids with oxidative decarboxylation of the pyruvic moiety. For direct production of the peptide by a similar mechanism, it is necessary to propose a condensation involving α -keto- δ -aminoadipic acid. Perhaps a more likely possibility is peptide bond formation by some simpler keto acid, or by an aldehyde, followed by a metathesis with glutamic acid. This latter mechanism is more attractive in relation to the more general problem of protein synthesis, since it eliminates the necessity for postulating a strange variety of α -keto acids, and, furthermore, the biological occurrence of many metathetical reactions has already been described; namely, the formation of glycogen from glucose

monophosphate (Cori and Cori (16)), the formation of polypeptides (Bergmann and Fraenkel-Conrat (17)), and phosphate transfer in general. The demonstration of the involvement of the imino radical in the formation of the peptide bond in this polypeptide and in the acetylation of aminophenylbutyric acid raises the question whether or not it is a necessary reactant in all peptide syntheses.

While the previous experiment provided evidence that α -iminoglutaric acid or a derivative thereof was used in the peptide synthesis, it did not establish that this was the only substrate used. More satisfactory evidence for the validity of the latter assumption would be the production of a peptide with an N^{15} content corresponding to the presence of a preponderance of "medium glutamic acid" (i.e., the original glutamic acid put into the medium). Accordingly, an experiment similar to the previous one was set up, but this time growth was interrupted in 48 hours with the expectation that in the early stages of growth there would not be so much newly synthesized glutamic acid available for peptide formation. Since the relative proportions of "medium" and synthesized glutamic acid present at the time of peptide formation would determine the ratio in which they would be incorporated into the peptide, the conditions obtaining in early growth should favor a high peptide content of "medium" glutamic acid and a low N^{15} value.

In this experiment the ammonia in the medium contained 6.99 atom per cent of N^{15} before and 6.82 atom per cent of N^{15} after growth. The solution of the acid-soluble copper-precipitable fraction had, after dialysis against citrate buffer and distilled water, an N^{15} content of 4.00 atom per cent. The glutamic acid hydrochloride (N found, 7.66 per cent; theory, 7.63 per cent) isolated from this peptide solution had 3.99 atom per cent N^{15} . This solution was designated as Preparation III in the earlier discussion concerning the purity of the peptide preparation at this stage. As stated in the previous discussion, it would be extremely unlikely, under the conditions of the experiment, that any amino acids other than the "medium" glutamic acid, would have an N^{15} content which did not correspond more closely to that of the ammonia than to that of the peptide.

From the results of this experiment, it can be seen that 42 per cent of the glutamic acid in the peptide was obtained from that previously present in the medium, confirming the expectation in regard to the composition of the peptide produced in early growth. It would seem, however, either that growth was not interrupted soon enough to fulfil the purpose of the experiment or that a new possibility must be considered; namely, that another substrate is used in alternation with the glutamic acid. In this case, analytical values corresponding to a peptide composition of 50 per cent "medium" glutamic acid would be the theoretical expectation in the absence of any

newly synthesized glutamic acid. Further studies are under way on this and other aspects of the problem that have been discussed.

SUMMARY

Studies have been carried out on the production of a polypeptide by a strain of *Bacillus subtilis* when grown on a medium of simple composition. Methods are described for the quantitative determination of the amount of peptide produced and for the preparation of the pure peptide.

It has been established that the peptide is composed solely of $d(-)$ -glutamic acid and evidence has been produced in favor of peptide linkage formation via the ω -carboxyl groups of the glutamic acid.

It has been found that the glutamic acid molecule skeleton can be used by the microorganism as a substrate for the formation of this peptide and that the reaction mechanism involves α -iminoglutaric acid or a derivative thereof.

The chemistry and physiology of this peptide will be the subject of further studies.

The author wishes to express his gratitude to Professor David Rittenberg of the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, for his assistance during the course of this study.

BIBLIOGRAPHY

1. Borsook, H., and Huffman, H. M., in Schmidt, C. L. A., The chemistry of the amino acids and proteins, Baltimore, 865 (1938).
2. Ivánovics, G., and Erdős, L., *Z. Immunitätsforsch. u. exp. Therap.*, **90**, 5 (1937).
3. Ivánovics, G., and Bruckner, V., *Z. Immunitätsforsch. u. exp. Therap.*, **90**, 301 (1937).
4. Hehre, E. J., and Sugg, J. Y., *J. Exp. Med.*, **75**, 339 (1942).
5. Schoenheimer, R., Ratner, S., and Rittenberg, D., *J. Biol. Chem.*, **130**, 703 (1939).
6. Ussing, H. H., *Acta physiol. Scand.*, **2**, 222 (1941); *Chem. Abst.*, **36**, 1959 (1942).
7. Ivánovics, G., *Zentr. Bakt., 1. Abt., Orig.*, **138**, 211 (1937).
8. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
9. Dakin, H. D., *J. Biol. Chem.*, **13**, 357 (1912-13).
10. Levene, P. A., and Bass, L. W., *J. Biol. Chem.*, **82**, 171 (1929).
11. Bovarnick, M., and Clarke, H. T., *J. Am. Chem. Soc.*, **60**, 2426 (1938).
12. Schoenheimer, R., and Ratner, S., *J. Biol. Chem.*, **127**, 301 (1939).
13. Keston, A. S., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **122**, 227 (1937-38).
14. Rittenberg, D., Ratner, S., and Hoberman, H. D., *J. Am. Chem. Soc.*, **62**, 2249 (1940).
15. du Vigneaud, V., Cohn, M., Brown, G. B., Irish, O. J., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, **131**, 273 (1939).
16. Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, **135**, 733 (1940).
17. Bergmann, M., and Fraenkel-Conrat, H., *J. Biol. Chem.*, **124**, 1 (1938).

OXIDATION-REDUCTION POTENTIALS MEASURED WITH THE DROPPING MERCURY ELECTRODE

IV. POLAROGRAPHIC STUDY OF α -OXYPHENAZINE

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The polarographic half wave potential of a reversible organic oxidation-reduction system in a well buffered solution represents the E'_0 of that system. This fact was first established by Müller and Baumberger (1) and has been verified repeatedly (2-4). The underlying theory, first developed by Heyrovský and Ilkovič (5), has been discussed many times and need not be described here. It was put to the most crucial tests by an extension to unbuffered solutions (6) and to compounds which are known to form semiquinones (7). The results were found to be in perfect agreement with theory in the unbuffered oxidation of hydroquinone (8, 9); the data obtained in the unbuffered reduction of quinone showed that some mathematical problems remain for complete analysis of the curves. The investigation of semiquinones demonstrated, in general, a fair agreement between theory and analytical data, but revealed some irregularities, so far unknown in polarography, which will be discussed in this paper.

Numerous organic compounds have been found which, under certain conditions, are oxidized or reduced in two univalent steps, as contrasted to the usual single bivalent reaction. The product of the first step in these reactions is an intermediate radical, called a semiquinone, the existence of which has been established colorimetrically, potentiometrically, and magnetometrically (10). Rosinduline GG was the first compound belonging to this group that was studied polarographically (1). It produced such complex curves that their interpretation remained questionable. A paper outlining the general applicability of the dropping mercury electrode to the detection of intermediate radicals (7) showed that curves obtained during the reduction of α -oxyphenazine were more reliable and that the polarographic data were in fair agreement with published results. Unfortunately, this was true only if the concentration of the dyestuff was sufficiently high. In dilute solutions of pH less than 3, there appeared a marked disproportionality in the two steps which theoretically should have been equal. This is best demonstrated by the polarograms shown in Fig. 1 which belong to the series previously reported (7). Increasing amounts of

α -oxyphenazine were added to 0.01 N nitric acid, and polarograms were made after each addition. The upper polarogram in Fig. 1 represents curves of the most dilute solution and shows clearly that the first step is larger than the second step. These curves were taken once with one-fifth of full galvanometer sensitivity (Curve A_1) to magnify the curve, and another time with one-twentieth of that sensitivity (Curve A_2), which is also used

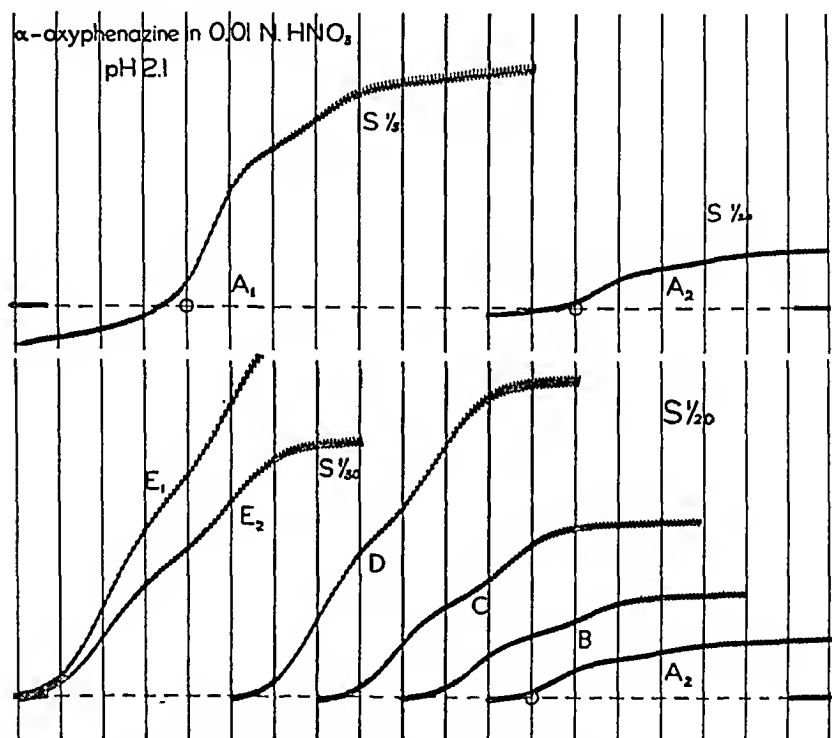
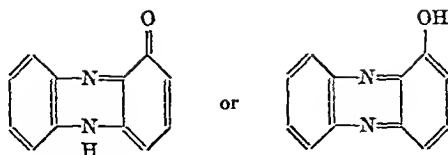


FIG. 1. Polarograms of α -oxyphenazine in 0.01 N HNO_3 ; Curve A 1.5, Curve B 2.7, Curve C 4.8, Curve D 8.7, and Curve E 10.5×10^{-4} M.

in the lower polarogram. Since the curve of the most concentrated solution (Curve E_1) failed to get on the record at this sensitivity, it was taken again with one-thirtieth of the galvanometer sensitivity (Curve E_2). Comparison of Curve E_2 with Curve A_1 makes obvious the effect of concentration on the ratio of the two steps in the reduction of α -oxyphenazine. This phenomenon was reproducible, and since it was contrary to expectations based on potentiometric observations, it was studied in greater detail.

α -Oxyphenazine

is well suited for these studies for several reasons. It is very stable over the whole pH range and even in concentrated sulfuric acid. It forms measurable amounts of semiquinone in a region of pH that can be conveniently investigated. Its solubility in the buffers used is of the same order in both the oxidized and leuco forms. A fairly concentrated solution can be prepared by adding an alcoholic solution of the dyestuff to the buffer. Finally, it is of biological importance, because it is formed during the alkaline decomposition of pyocyanine, one of the pigments produced by *Bacillus pyocyanus*.

The work presented in this paper was carried out with about 80 mg. of α -oxyphenazine, kindly presented by Dr. Leonor Michaelis of the Rockefeller Institute in New York. This compound of high purity had been synthesized and studied in detail in his laboratory (11, 12). Although the polarographic study was begun 3 years ago, the remaining sample still shows a sharp melting point of 155° (uncorrected), proving its stability.

In quantitative polarographic work, the Ilković equation (13) is used.

$$I_d = K \cdot D^{1/2} \cdot n \cdot C \cdot m^{1/2} \cdot t^{1/2}$$

Here I_d is the diffusion current, K is a constant, n is the number of electrons involved in the reduction of 1 molecule of the reducible substance, D is the diffusion coefficient of the reducible substance, C its concentration, m the weight of mercury flowing from the capillary per second, and t the time necessary for the formation of 1 drop of mercury. Thus the diffusion current must be proportional to the concentration of the reacting material as long as the same capillary is used for the analyses under identical conditions of temperature, mercury pressure, etc. Consequently, if the diffusion currents observed in the polarograms of Fig. 1 are plotted against the corresponding concentrations, a straight line should result which passes through zero if proper corrections are made for the residual current (14, 15). That this is actually the case for the diffusion current representing the complete two-step reduction may be seen from Fig. 2, Curve *c*. On the other hand, if each step is plotted by itself (Fig. 2, Curves *a* and *b*), a definite difference between them becomes apparent, decreasing with increasing concentration of the dye but never disappearing within the range

studied. This and the fact that both Curves *a* and *b* seem to end or undergo an abrupt change at a concentration of 10^{-4} M lead one to speculate that up to this concentration α -oxyphenazine exists exclusively in a form which cannot undergo a two-step reduction. For want of a better definition, this form will be called the "tautomer" for the rest of this discussion. At concentrations higher than 10^{-4} M the usual form of the dye makes its appearance and the concentration of the tautomer diminishes slightly.

The experimental arrangement was the same as that reported in the preceding publications (6, 7). Details about the theory and practice of polarography can be found in review papers (3, 16, 17) and books (14, 15, 18). The buffers used were prepared from c.p. materials and included citrate, phthalate, acetate, phosphate, and veronal buffers, besides free

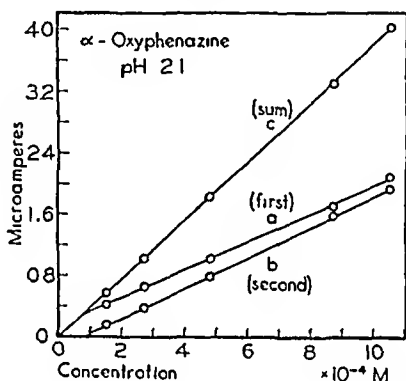


FIG. 2. Graph showing the relationship between wave heights and concentration of α -oxyphenazine.

sulfuric acid and potassium hydroxide. Their concentration was sufficient to insure adequate buffering for the reactions studied (6). The voltage increment between abscissas in all the polarograms published in this paper is 60 millivolts. Potentials are calculated with the abscissa marked with a circle as a point of reference, equal to the potential of the saturated calomel electrode. The horizontal dash line indicates the position of the galvanometer at rest. Before analysis, all solutions were freed from dissolved oxygen by a stream of either nitrogen or hydrogen gas.

Current Measurements

Polarograms of the different pure buffer solutions show no waves which can account for the observed discrepancies. An impurity in the α -oxyphenazine itself is also out of the question; if present, its concentration would have increased simultaneously with that of the dye. One might

think that the first step in the reduction is higher than the second step because the end-product (semiquinone) formed is unstable or diffuses away so rapidly that only a fraction of it remains for further reduction. This is highly improbable, because here, too, the disproportionality between the two steps would remain independent of concentration.

Alcohol seemed a more likely factor in the appearance of the tautomer wave. It was present because, for convenience, a concentrated solution of α -oxyphenazine in ethyl alcohol was added in small quantities to the aqueous buffers to prepare suitable concentrations of the dye. To invalidate the effect of alcohol, a few crystals of α -oxyphenazine were dissolved directly in 0.1 N sulfuric acid. The resulting solution was very dilute and produced only a single small wave on the polarogram. Addition of alcohol up to 10 per cent of this solution did not produce a second wave. On the other hand, leaving the aqueous solution in contact with excess dye crystals for over a month or grinding up the α -oxyphenazine with 0.1 N sulfuric acid produced more concentrated solutions with the resultant two unequal steps. These curves, too, remained practically unaltered by the addition of a little alcohol. The appearance of the tautomer wave, therefore, is not caused by the presence of small quantities of alcohol. A more detailed study of the effect of alcohol on the diffusion current and reduction potentials of α -oxyphenazine will be reported elsewhere.

Exclusion of the foregoing possibilities leaves polymerization as the next likely explanation. The problem of meriquinone formation and the special case of dimerization (19, 20) were first considered. Here symmetry between the two steps, or, in the case of more steps, constancy in the ratio of the steps, would be expected with a change in the concentration of the dye. Conversely, the separation of the steps should vary with concentration. Neither condition prevails in the present study.

Polymerization as found in the case of pyruvic acid (21) would require a proportional diminution of one wave with the simultaneous appearance of the polymer wave at higher concentrations. This, too, is not the case.

Finally, one might think of the possibility that 2 molecules of semiquinone formed in the first step of the reduction would react to produce 1 molecule of fully reduced and 1 molecule of fully oxidized α -oxyphenazine, thus increasing the first step and decreasing the second step, and that this reaction would for some unknown reason reach a limit. This hypothesis would require that the reverse situation should be found if fully reduced α -oxyphenazine were oxidized at the dropping mercury anode. In this case the oxidation at the more negative potential should produce the larger step; i.e., the second step should now be the larger one. Such a conclusion is easily tested and proved false, as may be seen in Fig. 3. The curves shown in this figure represent a solution of α -oxyphenazine in 0.1 N sul-

furic acid, reduced chemically to different stages by means of hydrogen gas in the presence of platinized asbestos (1). In order to produce these stages conveniently, additional amounts of platinized asbestos were added to the solution after each curve was taken. The color of the dye, indicating the oxidation levels (12), is stated for each curve. Fig. 3 demonstrates, first of all, the perfect reversibility of the system: the anodic curve is of the same shape as the cathodic curve and the half wave potentials are identical. It has been shown before (1, 8) that, whenever these conditions are fulfilled, the system is thermodynamically reversible. The slight slope of the line connecting the apparent half wave potentials is due to the IR drop in the cell. Fig. 3 also shows clearly that the first step in all curves is larger than the second step regardless of the original degree of oxidation of

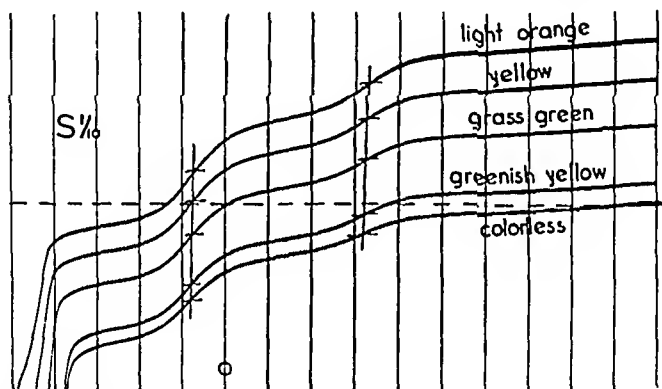


Fig. 3. Polarogram of α -oxyphenazine at different levels of oxidation, in 0.1 N H_2SO_4 . The electrode reaction is a reduction above, and an oxidation below the galvanometer zero (dash) line.

the solution. Actually the disproportionality between the two steps is even greater in the anodic curves than in the cathodic curves. This is due to a dilution of the solution caused by adsorption of the dye on the platinized asbestos, as indicated by the diminished total height of the two steps. As far as could be determined on the basis of other experiments, the diffusion coefficient of the leuco form of α -oxyphenazine is only slightly smaller than that of the oxidized form and may be considered identical for practical purposes.

The reverse test, oxidation of the fully reduced α -oxyphenazine by quinone, was also carried out in order to have a perfect analogy to potentiometric experiments (12). When carried out quickly, the experiments produced a set of curves similar to those in Fig. 3 and demonstrated again the perfect reversibility of the system. If the solutions were left standing

for some time, a slow reaction between oxidized α -oxyphenazine and quinone became apparent. This reaction is being investigated further.

Next, a series of experiments was performed to test the conclusion, based on Fig. 2, that only one step should be observed in sufficiently dilute solutions of α -oxyphenazine. This was found to be true, no matter whether the dyestuff was progressively diluted with solvent until it was no longer polaro-

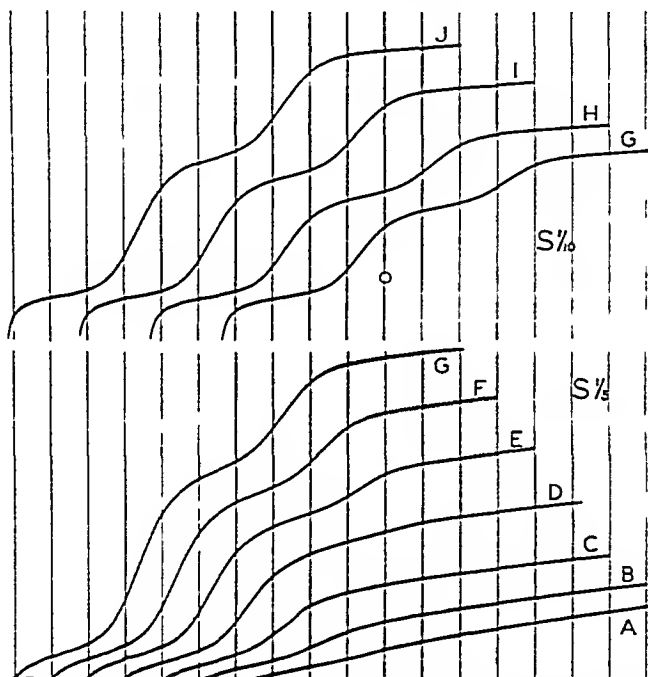


FIG. 4 Polarograms of α -oxyphenazine in 0.1 N H_2SO_4 , Curve A 0, Curve B 0.2, Curve C 0.5, Curve D 0.9, Curve E 1.3, Curve F 1.7, Curve G 2.1, Curve H 2.4, Curve I 3.1, and Curve J 3.7×10^{-4} M

graphically determinable, or whether an already dilute solution of the dye was added in increasing amounts to the pure solvent. An example of such a series is given by the polarograms shown in Fig. 4. Plotting of the data of this experiment results in a graph similar to Fig. 2 and again shows that only one wave exists when the concentration of the dye is less than 10^{-4} M. This single tautomer wave also was found to fulfil the aforementioned conditions for thermodynamic reversibility.

A corollary to this series of experiments was a concentration test. A very dilute solution of α -oxyphenazine in 0.1 N sulfuric acid (pH 1.35) was analyzed and showed only one wave on the polarogram (Fig. 5, A). This solution was then heated until about four-fifths of its former volume had evaporated, thus concentrating the α -oxyphenazine and the acid 5-fold. As was expected, two waves appeared on the polarogram, separated of course in accordance with the new medium which now has a pH of 0.65

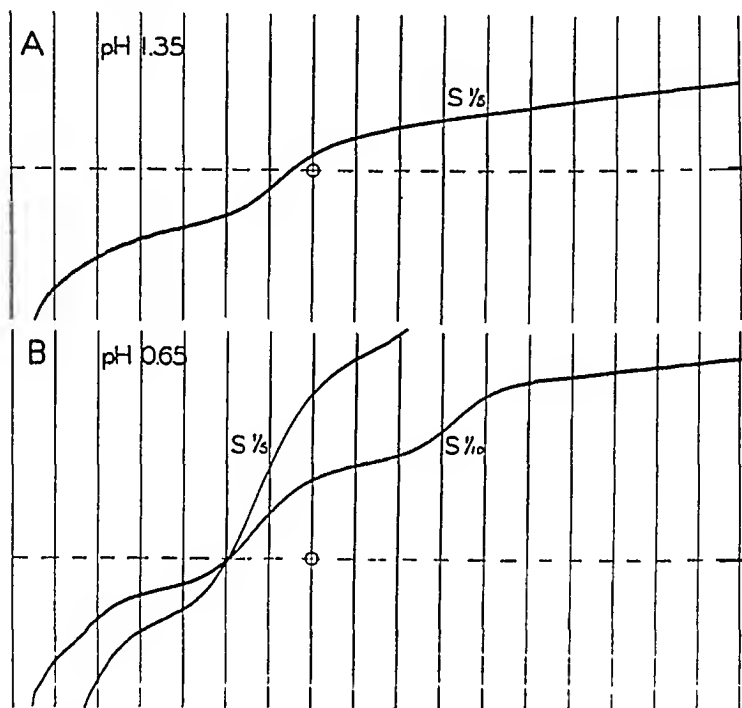


FIG. 5. A solution of 0.6×10^{-4} M α -oxyphenazine in 0.1 N H_2SO_4 (A); after 5-fold concentration by evaporation (B).

(Fig. 5, B). This experiment demonstrated conclusively that the single tautomer wave observed in dilute solutions must be due to α -oxyphenazine.

Potential Measurements

Besides the observation of the relative magnitude of the two steps in the reduction of α -oxyphenazine, there is the study of half wave potentials (5) useful in comparing polarographic and potentiometric data. In Fig. 6 are summarized the results of numerous polarographic determinations in different buffers covering practically the whole pH range. The half wave

potentials plotted here have all been corrected for IR (1, 8) and are referred to the normal hydrogen electrode. A temperature correction has also been made; so all values are for 30°. (A study of the temperature coefficients of the diffusion current and of the half wave potentials of α -oxyphenazine will be reported in a subsequent paper.) In Fig. 6 are likewise plotted the potentiometrically obtained values (for 30°) of Michaelis and

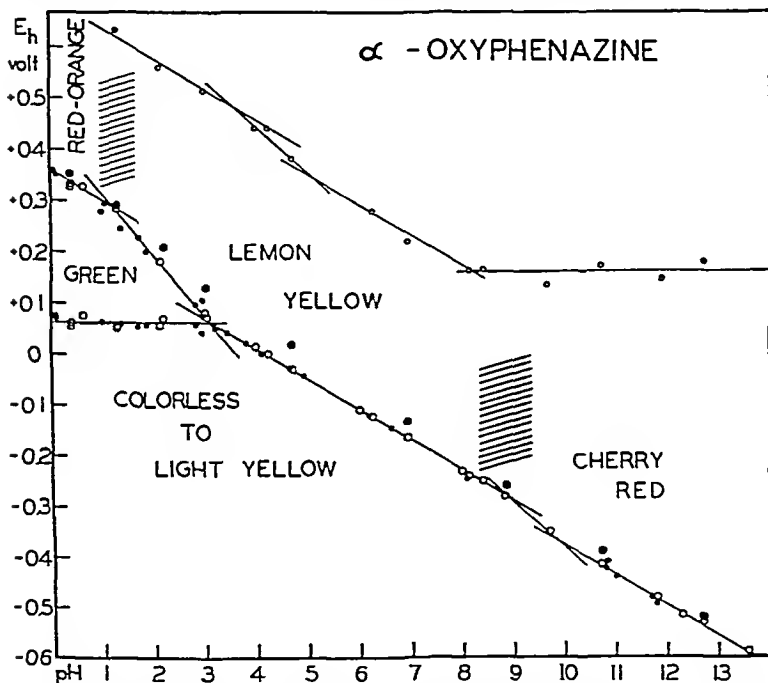


FIG. 6. Comparison of potentiometric and polarographic data. •, potentials determined potentiometrically by Michaelis and coworkers (11, 12). O, polarographic half wave potentials obtained with 5×10^{-4} and 10^{-3} M solutions. ●, polarographic half wave potentials obtained with 10^{-4} M and less concentrated solutions. ◦, polarographic half wave potentials of small anodic wave (see the text).

coworkers (11, 12). Since polarographic potentials are accurate only to about 10 millivolts (17), the agreement between the two types of measurement is excellent. There is one significant point of difference, however, in the placement of the second dissociation constant of the oxidized form of α -oxyphenazine. Michaelis, Hill, and Schubert (12) placed this at pH 10, while the polarographic evidence shows that it must be between pH 8 and

9. This is corroborated by a change in the color of the solution from yellow to cherry-red in the same pH range, determined with a glass electrode during a titration.

The polarographic half wave potentials marked by large open circles were obtained in solutions 5×10^{-4} and 10^{-3} M with respect to α -oxyphenazine. The large dots indicate the potentials of α -oxyphenazine in concentrations not exceeding 10^{-4} M. These show the influence of the tautomer which, between pH 3 and 11, appears to be more positive than the remaining α -oxyphenazine by about 30 to 60 millivolts. At the extreme ends of the pH scale this difference diminishes. Exact measurement of the tautomer half wave potential is difficult, since it shows a dependency on concentration.

The diffusion currents in all instances were measured by using the extrapolation method (14, 15). The residual current method could not be used to determine the diffusion current of α -oxyphenazine, since in all cases a small additional wave was found with a very positive potential (Fig. 7, Curve b; see also Fig. 5 (8)). This wave seems to be partly oxidative and partly reductive and is in no simple relation to the other waves observed. Further investigation is necessary before much can be said about it. For completeness, however, its approximate half wave potentials were also drawn into Fig. 6 with small circles.

One point should be mentioned with respect to Fig. 1 and similar polarograms presented in an earlier publication (7). They all were made with the same potassium nitrate-agar bridge, which must have been faulty. The potentials calculated from these polarograms, which did not fit any theoretical curve and could not be reproduced thereafter, can be explained only on the basis of a large diffusion potential and a high resistance of the bridge. Moreover, experiments performed in nitric acid are not as reliable as they first seemed, since marked changes in the curves were observed after the solution had stood for some time. Consequently all later experiments were carried out in sulfuric acid; the potentials thus obtained were in agreement with theory and with published potentiometric data. Detailed analyses of the curves revealed that the previously developed equation for the polarographic curve of semiquinone-forming compounds (7, 8, 22),

$$E = E_0 - \frac{RT}{2F} \ln \frac{I}{I_d - I} - \frac{RT}{2F} \ln \frac{\sqrt{4\kappa I_d^2 - (4\kappa - 1)(I_d - 2I)^2} - (I_d - 2I)}{\sqrt{4\kappa I_d^2 - (4\kappa - 1)(I_d - 2I)^2} + (I_d - 2I)}$$

holds if the concentration of the dye is large enough. A modification of this equation will have to be developed before it is applicable to dilute solutions with a prominent tautomer wave.

Effect of Drop Time

Normally, the effect of drop time of the mercury electrode on the diffusion current can be predicted from the Ilkovič equation. In the case of polarographic curves with maxima the influence of drop time is more marked (23), but such curves are not suitable for quantitative work. A study of α -oxyphenazine with different electrodes, under conditions in

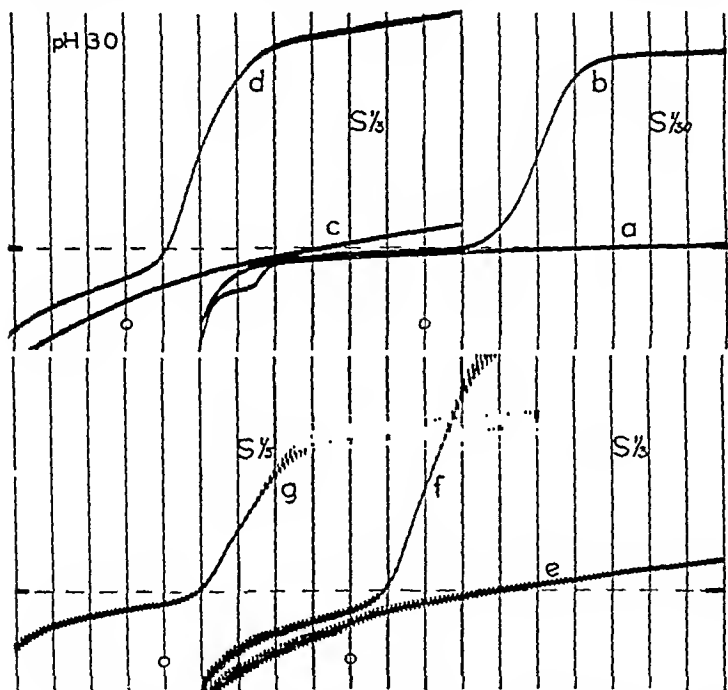


FIG. 7. Polarograms of α -oxyphenazine in McIlvaine's buffer, pH 3.0; Curve *a* 0, Curve *b* 0.001 M; Curve *c* 0, Curve *d* 0.0001 M; Curve *e* 0, Curve *f* 0.0001 M; and Curve *g* 0.0001 M. Capillary drop time, 1.35 seconds in the upper section, 4.1 seconds in the lower.

which no maxima are involved, brought out an effect of drop time not predictable from the Ilkovič equation and hitherto unknown. An example is shown in Fig. 7.

The top polarogram was made, like most polarograms shown in this paper, by using a fast capillary with a drop time of 1.35 seconds at a potential of -0.3 volt referred to the saturated calomel electrode. To determine the residual current, two curves were drawn (Curves *a* and *c*) with 9.0 cc.

of pure McIlvaine's buffer, pH 3.0, at two different galvanometer sensitivities. Then 1.0 cc. of 0.01 M α -oxyphenazine in 95 per cent ethyl alcohol was added and Curve *b* was obtained, superimposed on Curve *a*. This curve shows the previously mentioned small extra wave with the positive half wave potential which is largely oxidative. Next 1.0 cc. of this solution was added to 9.0 cc. of fresh buffer and Curve *d* was obtained with this 0.0001 M solution at the higher galvanometer sensitivity. This curve is superimposed on the pure buffer Curve *c*, and shows clearly that a small part of the extra wave with the positive potential must be reductive.

The curves in the lower section were obtained similarly but by using a slow capillary with a drop time of 4.1 seconds (at a potential of -0.3 volt referred to the saturated calomel electrode). Curve *e* represents pure McIlvaine's buffer at pH 3.0 and Curve *f* a 0.0001 M solution of α -oxyphenazine, superimposed at the same galvanometer sensitivity. Since this curve was not measurable, it was repeated at a lower galvanometer sensitivity (Curve *g*).

Comparison of the two waves of the upper polarogram shows that the relatively smooth S-curve of the concentrated solution (Curve *b*) is distorted in the more dilute solution into an unsymmetrical wave (Curve *d*). The curve of the same solution taken with the slow capillary (Curve *g*) looks quite different; two separate waves can be distinguished, of which one must be ascribed to the tautomer. The difference in the shape of the waves can mean only that the fast capillary produces a bigger tautomer wave which overlaps into the following wave. This conclusion was verified by studies in another medium, as demonstrated in Fig. 8. All the curves shown here were obtained with the same 0.0001 M solution of α -oxyphenazine in 0.1 N sulfuric acid at 24° . Three different capillaries were used, one of them at two different pressures of mercury, and so covered a range of drop times from 1.5 to 7.7 seconds. The galvanometer sensitivities were adjusted so that all curves could be measured with approximately the same accuracy. It becomes obvious from an inspection of the polarograms that the slower the drop time, the smaller the discrepancy due to the tautomer. In spite of all this, the sum of the diffusion currents of the two steps, the first one of which includes the tautomer, fulfils the requirements of the Ilkovič equation (13) fairly well, as will be seen from Table I.

In Table I, the capital letters refer to the curves of Fig. 8. In Columns 2 and 3 the number of the capillary used and its characteristic constant κ (15) are listed. Columns 4 and 5 give the pressure of mercury, P , and the corresponding drop time, t , at an applied voltage of -0.3 volt (referred to the saturated calomel electrode). Since $P/\kappa = m$ (15), the value $m^{1/2}t^{1/2}$ appearing in Column 6 can be calculated. The ratio between the average current and this factor must be a constant if the Ilkovič equation holds.

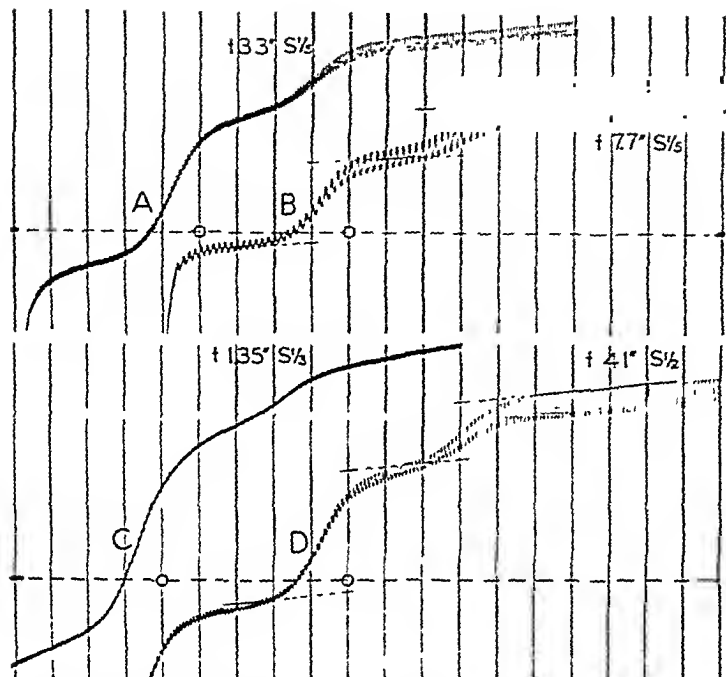


FIG. 8. Polarograms of 0.0001 M α -oxyphenazine in 0.1 N H_2SO_4 obtained with capillaries of different drop times (see the text).

TABLE I

Application of Ilkovič Equation to Diffusion Current of α -Oxyphenazine Measured with Different Dropping Mercury Electrodes

Curve (Fig 8)	Capillary No.	κ	P	t	$mI_d t$	Average current		Maximum current	
						I_d	$\frac{I_d}{mI_d t}$	I_d	$\frac{I_d}{mI_d t}$
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
			cm.	sec.	$\frac{mg. 2/3 \times sec.-1/2}{sec.-1/2}$	$\times 10^{-4}$ ampere		$\times 10^{-4}$ ampere	
A	1	29.7	64	3.3	2.042	55.6	27.2	56.6	27.7
B	1	29.7	29	7.7	1.380	36.4	26.4	40.9	29.6
C	2	44.6	74	1.53	1.506	39.1	26.0	39.2	26.1
D	3	182.0	83	4.1	0.748	20.1	26.9	20.7	27.7
Average.....						26.6 \pm 0.6			27.8

This *average* current is usually measured as illustrated in Curve B (Fig. 8). As may be seen from Column 8, the four results agree with an accuracy of ± 3 per cent. The diffusion coefficient, D , of α -oxyphenazine was then calculated. When *average* current values for I_d are used, the constant K in the Ilkovič equation (13) is equal to 605 if the current is expressed in microamperes, the concentration in mm per liter, and the weight of mercury in mg. (14). D is thus found to be $4.8 \pm 0.2 \times 10^{-6}$ cm.² sec.⁻¹ at 24°.

The Ilkovič equation should hold also if the *maximum* galvanometer deflections are measured, as indicated in Curve D (Fig. 8). The results given in Column 10, Table I, show, however, that this method yields much more diverse results. The diffusion coefficient, D , should also be determinable in this case by setting the constant K of the Ilkovič equation equal to 706 (14). This would give a value of D equal to 3.9×10^{-6} cm.² sec.⁻¹, which is definitely smaller than the value obtained with the *average* current. This difference is understandable, because the galvanometer

TABLE II
Effect of Drop Time on Relative Magnitude of Tautomer Wave

Curve (Fig. 8)	Capillary No.	t	Average current $\times 10^{-8}$ ampere				Tautomer
			1st wave (a)	2nd wave (b)	Total (a) + (b)	Tautomer (a) - (b)	
		sec.					per cent of total
A	1	3.3	39.1	16.5	55.6	22.6	41
B	1	7.7	23.0	13.4	36.4	9.6	26
C	2	1.53	32.2	7.0	39.2	25.2	64
D	3	4.1	14.0	6.1	20.1	7.9	39

used had a 5 second period of swing; so that it could never measure the actual maximum current, especially in the case of the fast electrodes. Although the *average* currents, likewise, are only approximations, and are very dependent on the characteristics of the galvanometer (15), the results thus obtained are more consistent and therefore more reliable. It should be pointed out, however, that the apparent *maximum* galvanometer deflection obtained with the fast No. 2 capillary can well be taken as the *average* current and as such substituted in the Ilkovič equation.

If the difference between the first and second steps of the curves of Fig. 8 is considered equal to the tautomer and expressed as per cent of the total diffusion current, as in Table II, the relationship between tautomer and drop time is brought out more clearly. When the results are plotted on a double logarithmic scale, as in Fig. 9, the points fit a straight line well enough to permit the following conclusions: Under identical experimental conditions, an electrode with a drop time of 0.7 second should produce

only the single tautomer wave, while an electrode with a drop time of at least 45 seconds should reduce the tautomer wave to 10 per cent of the total diffusion current.

A test of the first point is complicated by the increased residual current and by the fact that the dropping mercury electrode becomes less reliable with faster dropping rates. The second point indicates that no tautomer wave would be found if a stationary electrode were used. This is in agreement with potentiometric studies in which stationary platinum electrodes are used. The interesting problem remains, will a rotating platinum electrode (14) also produce a tautomer wave?

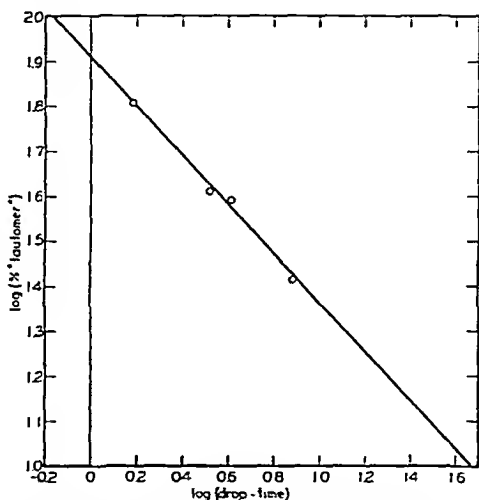


FIG. 9. Graph showing the relationship between drop time and the magnitude of the tautomer wave of $0.0001\text{ M } \alpha\text{-oxyphenazine}$ in $0.1\text{ N H}_2\text{SO}_4$.

CONCLUSION

This is the first time that a real difference has been found between polarographic and potentiometric results when reversible organic systems have been investigated. This difference may be of significance, since it has been found to exist in a number of dyestuffs similar to $\alpha\text{-oxyphenazine}$. It must not be supposed, however, that the appearance of the tautomer wave in dilute solutions is a peculiarity of the dropping mercury electrode. For instance, quinone, even at the lowest concentrations determinable, shows only a single wave with a constant half wave potential.

It would be premature to make many speculations about the nature of the tautomer or the significance of the observations. However, intra-

molecular rearrangements have sometimes been offered as an explanation of the sensitivity of certain dyestuffs to light and of their failure to obey Beer's law. Furthermore, such tautomers are often considered to be in an activated state. The more positive potential of the tautomer of α -oxyphenazine found in the present study would indicate that it could well be a representative of an activated complex which requires less energy to be reduced. This idea is strengthened by the additional fact that the concentration of the tautomer is always small and is even diminished by an excess of the normal dye molecules. Further experiments are in progress to accumulate additional information necessary before there can be a more serious attempt to explain the results.

SUMMARY

By means of the dropping mercury electrode, the existence of a tautomer of α -oxyphenazine with a more positive half wave potential has been demonstrated. Experiments show that this tautomer (a) does not form a semiquinone over the pH range 1 to 13, (b) is the only form in which α -oxyphenazine exists in solutions less concentrated than 10^{-4} M, (c) never exceeds this concentration but diminishes slightly with increasing concentration of α -oxyphenazine, and (d) is not altered by the presence of small quantities of ethyl alcohol.

The polarographic wave of this tautomer decreases with an increase in the drop time of the electrode. It is, therefore, impossible to show the existence of this tautomer with a stationary electrode. Thus, for the first time, a real difference has been found between potentiometric and polarographic data pertaining to reversible organic systems.

At sufficiently high concentrations, the effect of the tautomer on the half wave potential of α -oxyphenazine becomes negligible and the polarographic and potentiometric potentials show excellent agreement.

The author gratefully acknowledges his indebtedness to the late W. E. Benjamin and to Dr. John Staige Davis, Jr., for providing funds in support of this investigation.

Addendum—Since this manuscript went to press, an abstract has appeared of a related paper by Brdička and Knobloch (24) which, unfortunately, was unknown to me due to the war. In a polarographic study of riboflavin, these authors observed a small wave in acid solutions which was independent of the concentration of riboflavin and preceded the main reduction wave of this compound by 35 millivolts. In solutions less concentrated than 4×10^{-5} M only this extra wave with the more positive half wave potential remained. The structural similarity between riboflavin and α -oxyphenazine leaves little doubt that this is another case of the tautomerism reported in the present paper. A study of the effect of drop time is planned to verify this conclusion; it may also explain why Lingane and Davis (25) failed to observe the extra wave.

BIBLIOGRAPHY

1. Müller, O. H., and Baumberger, J. P., *Tr. Electrochem. Soc.*, **71**, 181 (1937).
2. Tachi, I., *Mem. Coll. Agric., Kyoto Imp. Univ.*, **42**, 27 (1938).
3. von Stackelberg, M., *Z. Elektrochem.*, **45**, 466 (1939).
4. Smith, L. I., Kolthoff, I. M., Wawzonek, S., and Ruoff, P. M., *J. Am. Chem. Soc.*, **63**, 1018 (1941).
5. Heyrovský, J., and Ilkovič, D., *Collect. Czechoslov. Chem. Communicat.*, **7**, 193 (1935).
6. Müller, O. H., *J. Am. Chem. Soc.*, **62**, 2434 (1940).
7. Müller, O. H., *Ann. New York Acad. Sc.*, **40**, 91 (1940).
8. Müller, O. H., in Cold Spring Harbor symposia on quantitative biology, Cold Spring Harbor, **7**, 59 (1939).
9. Kolthoff, I. M., and Orlemann, E. F., *J. Am. Chem. Soc.*, **63**, 664 (1941).
10. Michaelis, L., *Ann. New York Acad. Sc.*, **40**, 39 (1940).
11. Michaelis, L., *J. Biol. Chem.*, **92**, 211 (1931).
12. Michaelis, L., Hill, E. S., and Schubert, M. P., *Biochem. Z.*, **255**, 66 (1932).
13. Ilkovič, D., *Collect. Czechoslov. Chem. Communicat.*, **6**, 498 (1934).
14. Kolthoff, I. M., and Lingane, J. J., *Polarography*, New York (1941).
15. Müller, O. H., *The polarographic method of analysis*, Easton (1941).
16. Kolthoff, I. M., and Lingane, J. J., *Chem. Rev.*, **24**, 1 (1939).
17. Müller, O. H., *Chem. Rev.*, **24**, 95 (1939).
18. Heyrovský, J., in Böttger, W., *Physikalische Methoden der analytischen Chemie*, Leipzig, **2** (1936); **3** (1939).
19. Clark, W. M., Cohen, B., and Gibbs, H. D., *Pub. Health Rep., U. S. P. H. S.*, suppl. **54** (1926).
20. Michaelis, L., and Schubert, M. P., *Chem. Rev.*, **22**, 437 (1933).
21. Müller, O. H., and Baumberger, J. P., *J. Am. Chem. Soc.*, **61**, 590 (1939).
22. Brdička, R., *Z. Elektrochem.*, **47**, 314 (1941).
23. Heyrovský, J., *Actualités scientifiques et industrielles*, Paris, No. 90 (1934).
24. Brdička, R., and Knobloch, E., *Z. Elektrochem.*, **47**, 721 (1941); *Chem. Abstr.*, **36**, 4536 (1942).
25. Lingane, J. J., and Davis, O. L., *J. Biol. Chem.*, **137**, 567 (1941).

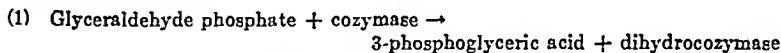
THE TWO-FOLD ACTIVATION OF CARBOHYDRATE BREAK-DOWN BY ARSENATE AND THE DEPHOSPHORYLATION OF PHOSPHOPYRUVIC ACID*

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Since Harden and Young discovered the enormous stimulation of the fermentation of hexose diphosphate by arsenate, this subject has repeatedly attracted the attention of investigators and every single step in the chain of intermediate reactions has been checked in regard to a possible activation by arsenate. It was found by Meyerhof (1) and Meyerhof and Kiessling (2) that in fermentation and glycolysis the first action of arsenate is on the transformation of triose phosphate to 3-phosphoglyceric acid. As was shown some years later by Meyerhof, Kiessling, and Schulz (3), Needham and Pillai (4), and Meyerhof, Ohlmeyer, and Möhle (5), glyceraldehyde phosphate is rapidly oxidized to phosphoglyceric acid by means of cozymase, if inorganic phosphate is simultaneously taken up and transferred by the adenylic system. However, in the presence of arsenate, the oxidation proceeds with equal speed, although phosphate is no longer taken up. Therefore, the oxidation-reduction in the presence of arsenate (cf. (3, 6)) is simply



Warburg and Christian (7) and Negelein and Brömel (8) showed that the reversible stoichiometric coupling reaction of Meyerhof, Ohlmeyer, and Möhle (5) could be traced to a phosphate uptake by glyceraldehyde monophosphate (Fischer-Baer ester), since they were able to isolate 1,3-diphosphoglyceric acid as the first oxidation product with cozymase. They assumed by analogy the formation of a similar arsenyl compound. But while diphosphoglyceric acid transfers its carbonyl phosphate to the adenylic system, the 1-arseno-3-phosphoglyceric acid is assumed to decompose spontaneously. This explanation of the effect of arsenate, although lacking direct experimental evidence, may be accepted as very probable.

However, there is a second action of arsenate. The end-product of

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reaction (1), 3-phosphoglyceric acid, is dephosphorylated via 2-phosphoglyceric acid and phosphopyruvic acid by way of the adenylic system (Lohmann and Meyerhof (9), Meyerhof and Kiessling (10)). Therefore, when phosphate acceptors are present to which the adenosine triphosphate can transfer its mobile phosphate groups, dephosphorylation proceeds with the same high speed as the formation of phosphoglyceric acid, but in the absence of such acceptors it is very much slower. In the presence of arsenate, however, the speed of dephosphorylation is very high in the absence of phosphate acceptors; so that the rapid fermentation of hexose diphosphate can run through to the end-products without any accumulation of phosphoglyceric acid. This second activation, as was shown several years ago (3), is a complicated reaction: dephosphorylation of phosphopyruvic acid in the presence of the phosphorylating enzyme system is by itself not activated by arsenate, but if catalytic amounts of the oxidation-reduction system, consisting of cozymase, triose phosphate, and active oxidative enzyme, are also added, dephosphorylation is 10 to 20 times enhanced by arsenate. In this case, therefore, both the phosphorylating and the oxidizing enzyme systems are necessary, as can well be demonstrated by the influence of iodoacetic acid, which suppresses the activation by arsenate, but does not check the normal phosphate transfer.

Pillai (11), using a dialyzed extract of acetone powder of muscle, made according to the method of Meyerhof and Ohlmeyer (12), confirmed these results and found at the same time that even in the absence of arsenate phosphoglyceric acid decomposes faster than might be expected, if there were no other way than the three consecutive steps,

(2) 2 Phosphoglyceric acid



2 phosphopyruvic acid + adenylic acid \rightarrow 2 pyruvic acid + adenosine triphosphate

(3) Adenosine triphosphate \rightarrow adenylic acid + 2 phosphate

The acetone-powder extract is, as is well known, practically free of adenylypyrophosphatase and reaction (3) can, therefore, not occur; but nevertheless, phosphoglyceric acid can be split in such an extract in the presence of a trace of adenosine triphosphate, and this dephosphorylation is suppressed by fluoride. The finding of Pillai was confirmed by Cori (13) and others, but so far the mechanism is unknown.

While we were engaged in the study of the enzymatic system in which cozymase reacts with glyceraldehyde phosphate in the presence of phosphate or arsenate, we made some observations which clear up the peculiar dephosphorylation of phosphopyruvic acid in the absence of adenylypyrophosphatase and the enormous enhancement of this reaction by arsenate.

Our investigations on the oxidation of glyceraldehyde phosphate with the pure enzyme system of Warburg and Christian will be published in the near future.¹ In the present paper we have restricted ourselves to the experiments of dephosphorylation, performed with acetone powder extract without further fractionation.

Methods and Preparations

Extract of rabbit muscle was precipitated with acetone. The dried powder was dissolved in water and dialyzed for 18 to 24 hours, as was described earlier (12). The dialyzing tubes, made from parlodion, were shaken in distilled water in a cold room. After dialysis the extract was again centrifuged. Usually 30 mg. of powder were used with 1 cc. of water, roughly corresponding to the original concentration of the fresh extract.

We used the crystallized acid barium salt of 3-phosphoglyceric acid prepared according to Meyerhof and Kiessling (15) from yeast. P, 8.3 per cent; $[\alpha]_D = -14^\circ$.

The phosphopyruvic acid was a synthetic preparation, prepared according to Kiessling (16) as the barium salt. The total P was 7.27 per cent, of which 1.45 per cent was inorganic and the remainder (5.82 per cent), phosphopyruvic P, measured by the iodine method.

The adenylyl pyrophosphate was the normal barium salt, prepared according to Lohmann (17); total P 8.05 per cent, inorganic P 0.75 per cent. The quotient (7' hydrolyzed P)/(organic P minus 7'-P), which has to be 2 in preparations of completely pure adenosine triphosphate, was lowered to 1.4 by allowing the sample to stand too long. Hence it contained a large amount of adenosine diphosphate, as could be proved by rephosphorylation with phosphopyruvic acid.

The cozymase was prepared by Ohlmeyer (18), and was originally 95 per cent pure in 1938. When used, it was still 55 per cent, measured manometrically by reduction with hydrosulfite. The adenylic acid (adenosine-5'-monophosphoric acid) was a crystallized preparation, obtained before the war by the courtesy of the Laokoon factory in Lwow, Poland.

For the phosphate determinations we used, as in former work, the method

¹ We may, however, use this opportunity to state that the dimeric diphosphoglyceraldehyde, recently prepared by Baer and Fischer (14) and kindly supplied for our experiments, is completely devoid of biological activity in the unpurified enzyme extracts as well as in the presence of cozymase and of the pure oxidizing enzyme. Only after the carboxyl phosphate is split up by hydrolysis with very weak HCl concentrations is the ordinary glyceraldehyde phosphate (old Fischer-Baer ester) obtained in monomeric form and then reacts with cozymase in the presence of phosphate or arsenate.

of Fiske and Subbarow (19) with the modifications of Lohmann and Jendrassik (20). Readings were made with a photoelectric photometer designed by Dr. David L. Drabkin in this department.² The special filter which was employed had a maximal transmission at λ 625 $m\mu$ of 1.36 per cent. The most suitable range of phosphate concentrations, with the Rubicon tubes S of 20 mm. diameter, lies between 0.05 and 0.1 mg. in 25 cc.; amounts between 0.02 and 0.2 mg. can still be accurately measured. The standard, closest to the unknown, must always be read between the measurements, since its color deepens slightly during a long series of measurements, about 1 per cent in half an hour, 2 to 3 per cent in 1 hour.

The determination of the different P fractions was made as in earlier work. Phosphopyruvic acid was determined according to the method of Lohmann and Meyerhof (9) and Meyerhof and Kiessling (10) by splitting off the phosphate by iodine in N NaOH; adenyl pyrophosphate was determined by 7 minutes hydrolysis in N HCl at 100°. If both are present simultaneously, the 7 minute value must be corrected, since 41 per cent of phosphopyruvic acid is split in this time. 3-Phosphoglyceric acid was determined by measuring the optical rotation in molybdate (21). It was not possible when phosphopyruvic acid was present to use the procedure of Pett (22) to remove arsenate, even if the temperature for incubation with sulfurous acid was lowered from 50° to 0°, since inevitably part of the phosphopyruvic acid decomposes. We, therefore, contented ourselves with using concentrations of arsenate not higher than 0.0015 M , about half the optimal concentration. Then when 0.3 to 0.5 cc. of the sample in 25 cc. was employed, the error introduced by arsenate was negligible, especially when the same amount of arsenate was added to the phosphate standard.

No advantage was found, for this and related work, in using any of the many modified procedures recently proposed for colorimetric P determination. If we had employed them, it would have been necessary to abandon the whole system of differential analysis by acid and alkaline hydrolysis. The modifications of the Fiske-Subbarow procedure by Lohmann and Jendrassik are important. By increasing the acidity from 0.5 N to 1 N H_2SO_4 the sensitivity to traces of silicic acid is removed, while with 0.5 N H_2SO_4 additional blue color develops after alkaline hydrolysis in glass vessels owing to dissolved traces of silicate. The incubation of the set of samples and standards for 7 minutes at 37° and subsequent cooling before matching are essential for stabilizing the color, which otherwise becomes practically constant only after some hours (*cf.* Berenblum and Chain (23)).

² This photometer was demonstrated by Dr. David L. Drabkin at the meeting of the American Society of Biological Chemists at Chicago, April, 1941, and will be described by him later.

For the purpose of the current experiments samples of 2 to 3 cc. were prepared, with 0.7 to 0.8 cc. of the extract made up from acetone powder. After a suitable interval, 1 cc. of 10 per cent trichloroacetic acid was added for deproteinization.

Results

Dephosphorylation of Phosphopyruvic Acid in Absence of Arsenate—After the discovery in Warburg's laboratory of 1,3-diphosphoglyceric acid and its phosphate transfer to adenosine diphosphate it seemed probable that the dephosphorylation of phosphoglyceric acid, independent of adenylypyrophosphatase, would proceed according to the following reactions.

- (2, a) Phosphopyruvic acid + adenosine diphosphate \rightarrow pyruvic acid + adenosine triphosphate
- (4) Adenosine triphosphate + 3-phosphoglyceric acid \rightleftharpoons adenosine diphosphate + 1,3-diphosphoglyceric acid
- (5) 1,3-Diphosphoglyceric acid \rightarrow 3-phosphoglyceric acid + phosphate
- (6) 3-Phosphoglyceric acid \rightleftharpoons 2-phosphoglyceric acid \rightleftharpoons phosphopyruvic acid

Reaction 5 would be a spontaneous, non-enzymatic decomposition, since according to Negelein and Brömel, a solution of diphosphoglyceric acid is unstable at all temperatures, more than 50 per cent decomposing in 30 minutes at 38° and pH 7.2.

The assumption of this sequence of reactions could be proved correct by applying to the system NaF, which blocks the enolase reaction between 2-phosphoglyceric and phosphopyruvic acid. It was demonstrated long ago that phosphoglyceric acid cannot be dephosphorylated in the presence of fluoride on account of this blocking. However, we find that when fluoride is present phosphopyruvic acid itself also is not dephosphorylated in our extracts with catalytic amounts of adenosine triphosphate. But this dephosphorylation is immediately started when phosphoglyceric acid in not too small amounts is added to this fluoride mixture. Although there is no interchange between phosphoglyceric and phosphopyruvic acids, owing to the blockage of the enolase (the phosphoglyceric acid remaining intact), phosphopyruvic acid decomposes now as rapidly as in the absence of fluoride. This, as can be seen from the equations, results from the dephosphorylation of adenosine triphosphate by the steps (4) and (5); so that adenosine diphosphate, necessary for reaction (2, a) is regenerated. The speed of the reaction is controlled by step (5).

In the muscle extract used here transphosphorylation of phosphopyruvic acid can take place as well with adenylic acid, according to equation (2), as with adenosine diphosphate. For example, in an enzymatic mixture similar to that of Tables I to III containing 0.05 N NaF, 600 γ of phosphopyruvic P, 310 γ of adenylic acid P, but no phosphoglyceric acid, in 30

minutes at 38° 525 γ of iodine-labile P disappeared and 575 γ of adenylic pyrophosphate P were formed; so the transphosphorylation was practically complete. Consequently the total chain of reactions can proceed with catalytic amounts of adenylic acid, although reaction (4) probably regenerates only adenosine diphosphate and no adenylic acid.³

The change of P, Tables I to IV, refers to inorganic phosphate (direct P), phosphopyruvic acid (iodine P), and labile P of adenosinepolyphosphoric acids (pyrophosphoric P).

From Table I it may be seen that in three experiments in the absence of phosphoglyceric acid, phosphopyruvic acid split off only 12 γ , 44 γ , 25 γ of P respectively; this value was increased by the addition of various amounts of phosphoglyceric acid to about 400 γ . This decomposition is not increased any more if fluoride is omitted (Samples 148-5 and 147-2), although phosphopyruvic acid is now rapidly regenerated by the enolase reaction. In samples containing fluoride the phosphopyruvic acid decreases as inorganic phosphate appears. That it decreases sometimes a little more than the inorganic phosphate increases is due to side reactions, but in the absence of phosphoglyceric acid it may be partly due to some leakage through the blocking of enolase, since the inhibition in the presence of 0.005 M inorganic phosphate is sometimes not quite complete with 0.1 N-0.05 N NaF.

Although Mg is necessary for the transphosphorylation (reaction (2)), its omission has very little effect here, probably because the dialysis of the extract for 20 hours is not exhaustive. Samples 147 and 149 demonstrate the necessity of the adenylic system, where adenylic acid and adenosine triphosphate are equivalent. That adenosine triphosphate by itself was practically stable in our enzymatic mixture was tested in three experiments, in which from 300 γ of pyrophosphate P in 1 hour at 38° only 25 γ , 12 γ , and 4 γ of P respectively were split off, while in half an hour, with an amount of an adenylic compound equivalent to 50 γ of pyrophosphoric P, 300 to 600 γ of P were split from phosphopyruvic acid.

Reaction (5), which is the last dephosphorylating step, is most probably non-enzymatic, since an enzyme which would split up the carbonyl phosphate would interfere with the rephosphorylation of the adenylic system according to equation (4), and this rephosphorylation is an essential step in the transfer of energy from oxidation-reduction to phosphorylation. This reaction, therefore, can proceed also under unphysiological temperatures and since the phosphorylating enzymes are, in general, not very sensitive to higher temperatures (*cf.* (25)) the dephosphorylation goes even a little faster at 55° than at 38°. This is shown in Table II.

³ We do not think that the occurrence of reaction (2) in addition to (2, a) can be explained by the reversible "myokinase reaction" of Kalekar (24), since the reaction starts with adenylic acid in the absence of higher phosphorylated stages.

The dephosphorylation depends upon the continuous regeneration of adenosine triphosphate by transphosphorylation with phosphopyruvic acid. Nevertheless, it seemed possible that also in the absence of phosphopyruvic acid adenosine triphosphate might, in part, break down with phosphoglyceric acid by reactions (4) and (5) if further reactions are inhibited by fluoride. But, apparently the equilibrium of reaction (4) is far to the left and our preparation of adenosine triphosphate contained an

TABLE I

Dephosphorylation of Phosphopyruvic Acid in Presence of Fluoride

Incubation 30 minutes at 38°. Samples 148 were made up to a total of 2.1 cc. with 0.8 cc. of extract, 0.1 cc. of equal amounts of NaHCO_3 and NH_4HCO_3 (4 per cent), phosphopyruvic acid with 480 γ of iodine P, and the following selective additions: 50 γ of pyrophosphoric P, 0.1 cc. of 0.1 N MgSO_4 , 0.1 cc. of N NaF. Phosphoglyceric acid contained 8.0 mg. of P per cc.

Samples 149 were made up to a total of 2.0 cc. with 0.7 cc. of extract, the same amount of bicarbonates, phosphopyruvic acid with 590 γ of iodine P, and the same selective additions, besides adenylic acid with 60 γ of adenylic P.

Samples 147 in 3 cc. with 1.0 cc. of extract. Phosphopyruvic acid with 700 γ of iodine P. Additions as in Samples 149.

Sample No.	Additions					Change of P	
	Mg	NaF	Pyrophosphoric P	Adenylic acid	Phosphoglyceric P	Direct P	Iodine P
					mg.	γ	γ
148-1	+	+	+	—		+12	-90
2	+	+	+	—	1.65	+330	-350
3	+	+	+	—	4.15	+355	-380
4	—	+	+	—	1.65	+265	-310
5	+	—	+	—	4.15	+350	+150
149-1	+	+	+	—		+44	-90
2	+	+	+	—	2.5	+390	-400
3	+	+	—	+	2.5	+440	
4	+	+	—	—	2.5	+66	
147-1	—	+	+	—		+25	
2	—	—	+	—	0.7	+410	
3	—	+	+	—	0.7	+450	
4	—	+	—	—	0.7	+70	

appreciable amount of adenosine diphosphate.⁴ We, therefore, never observed a decrease of pyrophosphoric P in such a system when inorganic phosphate was liberated. Actually, in such a system the pyrophosphoric P increased steadily until it nearly corresponded to the relation of 2 moles

⁴ Possibly with a 100 per cent pure preparation of adenosine triphosphate some decomposition could be demonstrated until the equilibrium of reaction (4) is attained.

of easily hydrolyzable to 1 mole of stable P. Consequently, adenosine triphosphate was regenerated while a little inorganic phosphate was split off. We must attribute this behavior to an incompleteness of the fluoride inhibition in the presence of high concentrations of adenylic compounds, although NaF was present in 0.1 N and 0.05 N in two experiments out of three and inorganic phosphate and Mg were added. (With 0.01 N NaF the inhibition was, of course, still more incomplete.) Cf. Table III.

Dephosphorylation of Phosphopyruvic Acid in Presence of Arsenate—Former experiments (3) have already demonstrated that the enhanced breakdown of phosphoglyceric acid in the presence of arsenate depends upon the activity of the oxidation-reduction system. It can easily be

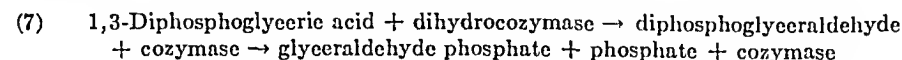
TABLE II

Influence of Temperature on Dephosphorylation

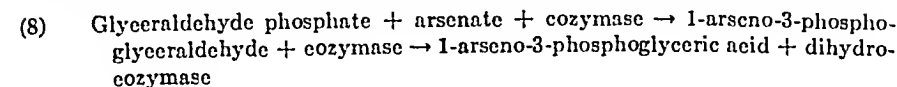
Samples 152 had the same composition as Samples 149 (Table I). Addition of 50 γ of pyrophosphoric P, 2.4 mg. of P of phosphoglyceric acid, 0.1 cc. of N NaF, 0.1 cc. of 0.1 N MgSO_4 . Dephosphorylation at 38° and 55°.

Sample No.	Time	Change of P					
		Direct P		Iodine P		Without extract, 55°	
		38°	55°	38°	55°	Direct P	Iodine P
	min.	γ	γ	γ	γ	γ	γ
152-1	5	+195	+222	-135	-177		
2	15	+375	+422	-290	-310		
3	30	+540		-440		0	-60

shown that the effect of arsenate results from the rapid removal of 1,3-diphosphoglyceric acid by the following reaction.



This reaction takes place also in the absence of arsenate. But in this case it is compensated for by the reverse reaction, so that an equilibrium results, by which phosphate is taken up as rapidly by glyceraldehyde phosphate in the presence of the enzymatic system as it is split up; hence with small amounts of all reactants no measurable turnover results. But this is not true in the presence of arsenate. In this case reaction (7) is not compensated for by the reverse reaction, because arsenate suppresses the phosphate uptake by glyceraldehyde phosphate, and according to the formulation of Warburg and Christian the oxidizing reaction is now



Since the arsenyl group would be spontaneously and rapidly split off, phosphoglyceric acid is liberated again and takes up phosphate according to reaction (4) and gets rid of this phosphate by reaction (7). While arsenate pushes the system continuously to the side of phosphoglyceric acid, the diphosphoglyceric acid unloads its carbonyl phosphate in the presence of catalytic amounts of dihydrocozymase by reaction (7).

To initiate reaction (7) some part of the cozymase must be reduced to dihydrocozymase. In yeast preparations not containing carboxylase this must be done by adding hexose diphosphate which gives rise to glyceralde-

TABLE III

Stability of Adenyl Pyrophosphate in Presence of Fluoride

Incubation 30 and 60 minutes at 38°. Samples were made up to a total of 2.0 cc. with 0.7 cc. of extract, 0.1 cc. of NaHCO_3 + NH_4HCO_3 (4 per cent), 0.1 cc. of MgSO_4 , 0.1 cc. of 0.1 M phosphate, adenyl pyrophosphate (mixture of adenosine tri- and diphosphate) containing about 300 γ of pyrophosphate P and about 200 γ of adenyllic P. Selective additions of NaF and phosphoglyceric acid.

Sample No	Additions		Time	Pyrophosphoric P		Change of direct P
	NaF	Phosphoglyceric acid		Total	Change	
		mg	min	γ	γ	γ
152-1	0.01		0	290		
2	0.01		60	328	+38	+4
3	0.01	2.5	0	290		
4	0.01	2.5	30	365	+75	+200
5	0.01	2.5	60	450	+160	+225
154-1	0.05		0	375		
2	0.05		60	350	-25	+12
3	0.05	1.65	0	375		
4	0.05	1.65	30	470	+95	+84
5	0.05	1.65	60	495	+120	+105
155-1	0.1	1.65	0	255		
2	0.1	1.65	30	354	+99	+60
3	0.1	1.65	60	335	+80	+122

hyde phosphate by the action of zymohease. In muscle preparations addition of hexose diphosphate is not necessary, since pyruvic acid formed from phosphopyruvic can reduce the cozymase and start reaction (7).

To prove this reaction mechanism, we introduced NaF into the system as in the above experiments. Then arsenate has no dephosphorylating effect even in the presence of the oxidation-reduction system as long as the fluoride blockage of the enolase persists.⁵ Adding phosphoglyceric

⁵ In experiments of longer duration, 45 minutes to 1 hour, some leakage can occur through the NaF barrage, so that traces of phosphoglyceric acid are formed. These suffice to start the reaction cycle (7) and (8), by which phosphopyruvic acid is rapidly decomposed.

acid permits the activation by arsenate to take place. Poisoning with iodoacetic acid destroys it again.

With the optimal concentration of arsenate of 0.005 M in yeast maceration juice the dephosphorylation can be enhanced 20 times; in muscle extract, about 6 to 10 times. For technical reasons we contented ourselves with concentrations of 7×10^{-4} to 1.5×10^{-3} of As. With the amount of enzyme used here the rate increased 3 to 4 times. These experiments were made at 20°, since at 38° even without arsenate, 80 per cent of the added phosphopyruvic acid was decomposed in 30 minutes with the addition of phosphoglyceric acid. As can be seen in Table IV, arsenate increases the speed of dephosphorylation with added phosphoglyceric acid from 99 γ of P in 10 minutes to 375 γ (Samples 155-1 and 155-3), from 138 γ of P in 20 minutes to 440 γ (Samples 155-2 and 155-4). The effect of phosphoglyceric acid on this rate may be seen from Samples 156, 153, and 158. In 15 and 20 minutes without this acid, 0 γ and 24 γ of P are split off from phosphopyruvic acid; with addition of 84 γ of P of phosphoglyceric acid, 90 γ of inorganic P are formed; with 165 γ of P of phosphoglyceric acid, 255 γ of inorganic P; with addition of 1.65 mg. of P, 350 γ of inorganic P are formed. As Sample 156 shows, no P is split off in the absence of arsenate without phosphoglyceric acid in 45 minutes; with 165 γ of P of phosphoglyceric acid added, 65 γ of P are split off; with 1.65 mg. of P added, 170 γ of P are split off. But in the presence of arsenate in 15 minutes with 165 γ of P of phosphoglyceric acid, 159 γ of P are split off; and with 1.65 mg. of P of phosphoglyceric acid, 187 γ of inorganic P are split off. Here the difference for different concentrations of phosphoglyceric acid is small, since reaction (5), the spontaneous decomposition of the diphospho ester, plays no rôle.

The participation of the oxidation-reduction system may be seen from the samples with added iodoacetic acid (Samples 153-8 and 155-5) in which the dephosphorylation is exactly equal to that in the absence of arsenate. (The experiments in the absence of cozymase have less significance, since cozymase is incompletely removed by 20 to 24 hours dialysis (*cf.* (12).)

Since we have found that glutathione preserves the activity of the purified oxidizing enzyme (*cf.* also (26)), we added glutathione to Samples 156-6, 156-10, and 158-6 and found that the rate of dephosphorylation in the presence of arsenate was somewhat enhanced.

In Sample 158 the phosphate concentration was increased to 0.11 M and the fluoride to 0.11 N, to secure a complete inhibition of enolase. Although the P determinations are a little less accurate because of the high initial phosphate, a still more striking difference is now visible between the results with fluoride and those without. With fluoride in the absence of phosphoglyceric acid, no phosphate is split off in the presence of arsenate, while

TABLE IV

Effect of Arsenate on Dephosphorylation of Phosphopyruvic Acid in Presence of Fluoride

Incubation at 20°. Samples were made up to a total of 2.0 to 2.2 cc. with 0.7 to 0.8 cc. of extract, 0.05 cc. of $\text{NaHCO}_3 + \text{NH}_4\text{HCO}_3$, 0.05 to 0.1 cc. of 0.1 N MgSO_4 , 0.05 cc. of 0.1 M phosphate (in Sample 153, 0.5 M), 0.05 to 0.1 cc. of 0.02 M ascorbic acid, phosphopyruvic acid with 590 γ of iodine P, and selective additions of arsenate, fluoride, 0.10 mg. of cozymase, and phosphoglyceric acid with various amounts of P. In two samples iodoacetate 1:1500 was added to the extract 30 minutes prior to the beginning of the experiment; in three others 0.1 cc. of 0.1 N glutathione was added.

Sample No.	Additions					Time	Change of P	
	Arsenate	NaF	Cozy-mase	Phospho-glyceric P	Iodoacetate		Direct P	Iodine P
	M	N		$mg.$		$min.$	γ	γ
153-1		0.05	+	1.65		20	+153	-210
2	7.5×10^{-4}	0.05	+			20	+24	
3	7.5×10^{-4}	0.05	+	0.084		20	+90	
4	7.5×10^{-4}	0.05	+	1.65		5	+111	-160
5	7.5×10^{-4}	0.05	+	1.65		20	+350	-380
6	7.5×10^{-4}		+	1.65		20	+370	-120
7	7.5×10^{-4}	0.05	-	1.65		20	+170	
8	7.5×10^{-4}	0.05	+	1.65	+	20	+145	
155-1		0.1	+	1.65		10	+99	
2		0.1	+	1.65		20	+138	-190
3	1.0×10^{-3}	0.1	+	1.65		10	+375	-350
4	1.0×10^{-3}	0.1	+	1.65		20	+440	-420
5	1.0×10^{-3}	0.1	+	1.65	+	20	+123	-200
6	1.0×10^{-3}	0.1	-	1.65		20	+265	-309
					Glutathione			
156-1		0.09	+			45	0	-70
2		0.09	+	0.165		45	+65	-155
3		0.09	+	1.65		45	+170	-203
4	1.0×10^{-3}	0.09	+	0.165		15	+159	-198
5	1.0×10^{-3}	0.09	+	1.65		15	+187	-240
6	1.0×10^{-3}	0.09	+	1.65	5×10^{-3}	15	+265	-270
7	1.0×10^{-3}	0.09	+	*		45	+297	-400
8	1.0×10^{-3}	0.09	+	0.165		45	+435	
9	1.0×10^{-3}	0.09	+	1.65		45	+420	-460
10	1.0×10^{-3}	0.09	+	0.165	5×10^{-3}	45	+463	-475
158-1		0.11	+	1.65		15	0	
2	1.5×10^{-3}	0.11	+			15	0	0
3	1.5×10^{-3}		+			15	+336	-355
4	1.5×10^{-3}	0.11	+	0.165		15	+255	-260
5	1.5×10^{-3}		+	1.65		15	+350	0
6	1.5×10^{-3}		+	1.65	5×10^{-3}	15	+327	

* See foot-note 5.

without fluoride, 336 γ of P are split off (Sample 158-3); this amount is not increased even with a large quantity of phosphoglyceric acid added (Sample 158-5), although here phosphopyruvic acid is regenerated.

That phosphoglyceric acid really plays a catalytic rôle as phosphate carrier could be ascertained by polarimetric measurements in the presence of molybdate. The added small amounts of phosphoglyceric acid remain constant. For example, in Samples 156-4 and 156-8 the rotations were $\alpha = -0.557^\circ$ and -0.535° . The expected rotation, after the establishment of the equilibrium $3\text{-phosphoglyceric} \rightleftharpoons 2\text{-phosphoglyceric}$ acid was $\alpha = -0.61 \times 0.9 = -0.55^\circ$. At the same time nearly 3 times this amount of phosphopyruvic acid were decomposed.

DISCUSSION

The enzymatic system investigated in this paper concerns the last phosphorylated compound in the chain of intermediaries in glycolysis and fermentation. Normally the dephosphorylation of phosphopyruvic acid proceeds rapidly on account of the phosphate acceptors present: glucose, hexose monophosphate, and creatine. On the other hand, in the absence of such acceptors, the dephosphorylation is generally brought about by means of the adenylypyrophosphatase according to equations (2) and (3). Our present experiments explain how dephosphorylation is possible in the absence of stable phosphate acceptors and of adenylypyrophosphatase, a fact first clearly shown by Pillai. They throw light, moreover, on a related fact observed some years earlier, that, in the absence of arsenate and of phosphate acceptors, phosphoglyceric acid ferments 2 to 4 times as fast as does hexose diphosphate (*cf.* (1)). Fermentation takes place by way of phosphoglyceric acid and, as can be shown, no intermediary step between the two esters is responsible for the relative delay in the fermentation rate of hexose diphosphate as compared with phosphoglyceric acid. It follows from the present experiments that the true reason for this difference is the *concentration* of phosphoglyceric acid. As an intermediary in the breakdown of hexose diphosphate it is formed by reaction (4), going from right to left. It is then immediately removed by reactions (6) and (2); so that the concentration remains very low. Inorganic phosphate is regenerated by means of the adenylypyrophosphatase. (The possibility of a partial decomposition of hexose diphosphate to hexose monophosphate, as described by Lohmann, is not relevant in this connection (27).) On the other hand, if phosphoglyceric acid is added in high concentration, then reaction (4) is enabled to go from left to right, and this reaction together with reaction (5) adds to the other dephosphorylation and increases the rate of formation of inorganic phosphate. We think that this type of dephosphorylation has no physiological importance as an intermediary reaction.

But this is different in the presence of arsenate. Here hexose diphosphate ferments with the same high speed as does phosphoglyceric acid, a fermentation rate identical with the normal rate of fermentation of sugar. Our experiments show that very low concentrations of phosphoglyceric acid are already active, and the differential amount existing during the continuous breakdown of hexose diphosphate is, therefore, sufficient. This results from the removal of the 1,3-diphosphoglyceric acid by biochemical reduction (reaction (7)). Under suitable conditions reactions (8) and (7) compensate each other and the system functions in combination with reactions (4) and (2) for the rapid splitting of phosphopyruvic acid. In the over-all breakdown starting with hexose diphosphate, reaction (8) must proceed with higher speed than reaction (7), since otherwise glyceraldehyde phosphate would not be oxidized at all. This difference follows from the fact that only part of the phosphoglyceric acid reacts according to reaction (4) and forms diphosphoglyceric acid, while at the same time the other part disappears via phosphopyruvic and pyruvic acids.

The proof of the reactions formulated above depends, of course, upon the existence of the 1,3-diester. So far only the 1,3-diphosphoglyceric acid has been experimentally demonstrated by Negelein and Brömel. We will shortly report experiments in which the presence and the concentration of a diphosphorylated intermediary between glyceraldehyde phosphate and diphosphoglyceric acid can be demonstrated. Although this formation depends upon the presence of an oxidizing enzyme and cozymase, it is probably the 1,3-diphosphoglyceraldehyde of Warburg and Christian. But even if these reactions were still more complex, the mechanism of the dephosphorylation described in this paper seems definitely established.

SUMMARY

1. The enzymatic dephosphorylation of phosphopyruvic acid in the absence of stable phosphate acceptors and of adenylypyrophosphatase, but in the presence of catalytic amounts of the adenylic system, is due to the spontaneous breakdown of 1,3-diphosphoglyceric acid formed by phosphate transfer from adenosine triphosphate to 3-phosphoglyceric acid.

2. The many fold acceleration of this dephosphorylation by arsenate in the presence of the same system plus the oxidation-reduction system is due to the reduction of 1,3-diphosphoglyceric acid, yielding glyceraldehyde phosphate and inorganic phosphate, while the counterbalancing oxidation of glyceraldehyde phosphate proceeds in the presence of arsenate without uptake of inorganic phosphate.

BIBLIOGRAPHY

1. Meyerhof, O., *Biochem. Z.*, 273, 80 (1934).
2. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, 280, 99 (1935).

3. Meyerhof, O., Kiessling, W., and Schulz, W., *Biochem. Z.*, **292**, 25 (1937).
4. Needham, M. D., and Pillai, R. K., *Biochem. J.*, **31**, 1837 (1937).
5. Meyerhof, O., Ohlmeyer, P., and Möhle, W., *Biochem. Z.*, **297**, 90, 113 (1938).
6. Meyerhof, O., *Ergebn. Physiol.*, **39**, 10 (1937).
7. Warburg, O., and Christian, W., *Biochem. Z.*, **301**, 22 (1939); **303**, 40 (1939).
8. Negelein, E., and Brömel, H., *Biochem. Z.*, **301**, 135 (1939); **303**, 132 (1939).
9. Lohmann, K., and Meyerhof, O., *Biochem. Z.*, **273**, 60 (1934).
10. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **281**, 249 (1935).
11. Pillai, R. K., *Biochem. J.*, **32**, 1087 (1938).
12. Meyerhof, O., and Ohlmeyer, P., *Biochem. Z.*, **290**, 334 (1937).
13. Cori, C. F., in A symposium on respiratory enzymes, Madison, 186 (1942).
14. Baer, E., and Fiseher, H. O. L., *J. Biol. Chem.*, **143**, 563 (1942).
15. Meyerhof, O., and Kiessling, W., *Biochem. Z.*, **267**, 313 (1933).
16. Kiessling, W., *Ber. chem. Ges.*, **69**, 2331 (1936).
17. Lohmann, K., *Biochem. Z.*, **254**, 381 (1932).
18. Ohlmeyer, P., *Biochem. Z.*, **297**, 66 (1938).
19. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
20. Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419 (1926).
21. Meyerhof, O., and Schulz, W., *Biochem. Z.*, **297**, 60 (1938).
22. Pett, L. B., *Biochem. J.*, **27**, 1672 (1933).
23. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 286, 295 (1938).
24. Kalekar, H. M., *J. Biol. Chem.*, **143**, 299 (1942).
25. Curtius, L., and Ohlmeyer, P., *Biochem. Z.*, **298**, 41 (1938).
26. Rapkine, L., *Biochem. J.*, **32**, 1729 (1938).
27. Lohmann, K., *Biochem. Z.*, **262**, 137 (1933).

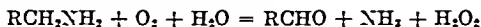
OXYGEN CONSUMPTION DURING THE HISTAMINE-HISTAMINASE REACTION

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According to Zeller (1, 2) the action of histaminase (diamine oxidase) is represented by the following reaction.



When he employed crude extracts of pig kidney, Zeller found that 2 and sometimes more atoms of oxygen were used up for every mole of substrate oxidized. Kiese (3) found that with crude extracts 2 atoms of oxygen were used per mole of histamine, but that after the histaminase had been repeatedly precipitated with ammonium sulfate between 30 and 60 per cent saturation only 1 atom of oxygen was used. He found the production of ammonia, however, to be 1 mole per mole of histamine, no matter whether crude or purified histaminase was employed. Kiese states that his ammonia determinations were not very reliable, owing to high blanks.

Zeller (4) employed inhibitors and found that the addition of those which affected the iron-containing enzymes decreased oxygen consumption. He believed that the 1st atom of oxygen was used in the deamination (real diamine oxidase) and that the 2nd was probably used for further oxidation of the deaminized product (suspected iron-containing enzyme).

EXPERIMENTAL

The author has found that when crude kidney extracts act upon histamine more than 2 atoms are used per mole of histamine and that the oxygen uptake does not cease within a reasonable length of time, due to exhaustion of the substrate, although the rate of oxygen uptake does diminish. Purified histaminase that was obtained by ammonium sulfate fractionation was found to cause the uptake of less oxygen than crude histaminase.

The histaminase employed in Experiments 1 and 2 for which curves are shown in Figs. 1 and 2 was prepared as follows: The cortex of fresh pig kidney was minced in a meat grinder, mixed with an equal volume of 2.5 per cent sodium chloride, homogenized in a milk mixer (Powermaster), heated to 62° for 10 minutes, and then centrifuged in a Swedish angle centrifuge. The oxygen consumption was determined in the Warburg

apparatus at pH 7.2 (phosphate buffer) with air at 38°. The substrate was 1 mg. of histamine dihydrochloride. This crude histaminase was purified by adding ammonium sulfate to 20 per cent saturation and discarding the precipitate. The filtrate was brought to pH 5.5 with acetate buffer and solid ammonium sulfate was added to this to make it 50 per cent saturated. The precipitate formed was filtered off. It was called Preparation P₆₀. The filtrate was brought to 80 per cent saturation and this precipitate was filtered off and called Preparation P₈₀. When tested by itself, P₈₀ was entirely inactive towards histamine, but it markedly increased the activity of Preparation P₆₀. The color of solutions of Preparation P₈₀ was red and oxyhemoglobin was present, as shown by absorp-

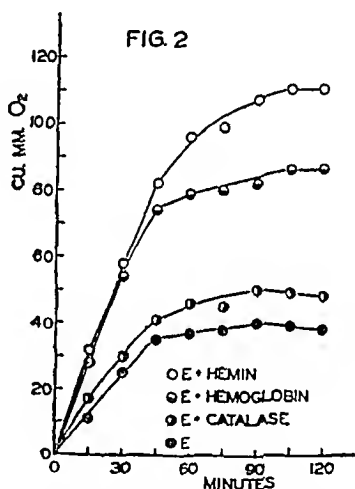
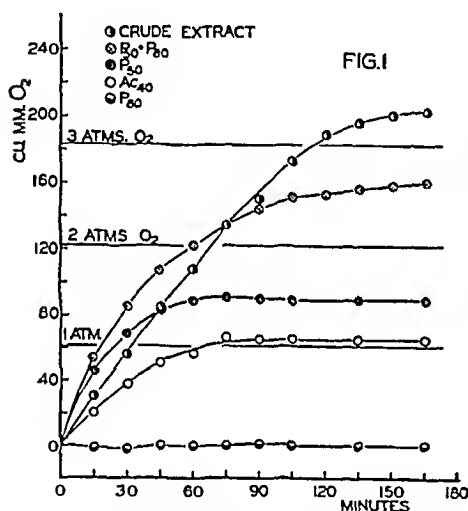


FIG. 1. Oxygen consumption of histaminase preparations of increasing purity.

FIG. 2. Oxygen consumption of purified Enzyme E alone and after the addition of different hemin compounds.

tion bands. During the tests in which Preparations P₆₀ and P₈₀ were used together, the red color changed to brown.¹ Preparation P₆₀ was still further purified by dialyzing against 2.5 per cent sodium chloride and precipitating by the addition of acetone to 40 per cent concentration. The precipitate was dissolved in 2.5 per cent sodium chloride and was called Preparation Ac₄₀. Preparation Ac₄₀ was buffered with acetate to pH 5.5 and adsorbed on Ca₃(PO₄)₂. The enzyme was eluted with M/15 phosphate buffer, pH 7.2. The elution was called Enzyme E; it was faintly yellow and contained only traces of catalase.

¹ The oxidation of hemoglobin to methemoglobin was observed by Zeller (1), but oxygen consumption was not investigated.

The histaminase used in Experiment 3 (Fig. 3) was purified essentially by the same steps but in a different order. The crude extract was first precipitated with acetone, then adsorbed on $\text{Ca}_3(\text{PO}_4)_2$, and eluted, and finally fractionated with ammonium sulfate.

The results obtained in Experiment 1 suggest that apparent oxygen consumption depends on the presence of hemoglobin. This was confirmed by Experiment 2. The addition of hemoglobin, or of hemin, to the purified Enzyme E markedly increased the oxygen consumption. Neither hemoglobin nor hemin, when incubated alone with histamine, showed any detectable oxygen consumption.

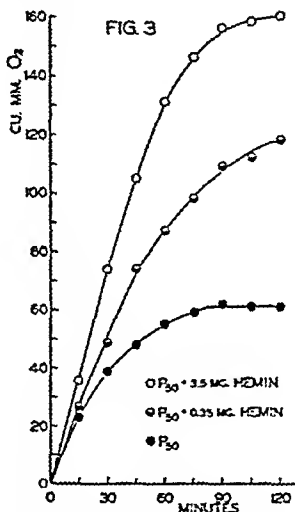


Fig. 3. Effect of different amounts of hemin on the oxygen consumption of purified histaminase.

In Experiment 3 different quantities of hemin (0.1 and 1 mole per mole of histamine) were added to the purified histaminase. The results showed that with increased amounts of hemin the oxygen uptake was also increased; however, no stoichiometric relation could be detected.

Zeller's conclusion that H_2O_2 is formed during the reaction is based on the indirect oxidation of alcohol. In the crude non-heated preparations, as used by Zeller, the amount of catalase was very high. Added hydrogen peroxide was decomposed within a few minutes. Heating to 62° destroyed a considerable amount of catalase, but even the purified extracts contained detectable amounts of catalase. The addition of crystalline beef liver

catalase to the purified Enzyme E, almost free from catalase (Fig. 2), by no means decreased the oxygen consumption, which would have been the case if H_2O_2 had been formed.

In order to test the validity of the method of coupled alcohol oxidation, crude unheated extract was used. Minced kidney cortex was extracted with 2 volumes of 2.5 per cent sodium chloride, centrifuged, and filtered. The findings of Keilin and Hartree (5) that H_2O_2 , when added to the system, does not oxidize alcohol were confirmed. The results of an experiment in which alcohol and histamine were used as substrates separately and together are shown in Table I. The oxygen consumption in the vessel (Flask IV) containing both histamine and alcohol was of the same order of magnitude as the sum of the oxygen consumed in the vessels (Flasks II and

TABLE I
Oxygen Consumption When Alcohol and Histamine Are Used As Substrates

	Flask I	Flask II	Flask III	Flask IV
	Main vessel			
	2.0 ml. kidney extract, 0.3 ml. buffer, 0.7 ml. saline	2.0 ml. kidney extract, 0.3 ml. buffer, 0.6 ml. saline	2.0 ml. kidney extract, 0.3 ml. buffer, 0.6 ml. saline	2.0 ml. kidney extract, 0.3 ml. buffer, 0.5 ml. saline
	Side arm			
	0.1 ml. H_2O_2 , 0.1 M	0.1 ml. H_2O_2 , 0.1 M; 0.1 ml. alcohol, M	0.1 ml. H_2O_2 , 0.1 M; 0.1 ml. = 1 mg. histamine dihydrochloride	0.1 ml. H_2O_2 , 0.1 M; 0.1 ml. alcohol, M; 0.1 ml. = 1 mg. histamine dihydrochloride
	<i>c.mm. O₂</i>	<i>c.mm. O₂</i>	<i>c.mm. O₂</i>	<i>c.mm. O₂</i>
5 min. after mixing	+119.4	+109.8	+110.0	+106.7
5 hrs.....	-61.1	-117.6	-148.5	-209.5
Corrected for blank.	0	-56.5	-87.4	-148.4

III) with alcohol alone and with histamine alone. In other experiments of the same type the oxygen consumed with two separate substrates accounted for 75 to 95 per cent of the oxygen consumed with both substrates together. In all experiments the vessel containing alcohol showed higher oxygen consumption than the blank, but in the case of extracts which were previously stored a few days in the refrigerator, the oxygen uptake in the vessel with alcohol ceased earlier than in other vessels.

The results of these experiments indicate that oxidation of alcohol might be due to the action of alcohol dehydrogenase, or else it might be coupled with some other oxidizing reaction occurring in the blank itself. The application of this method for detection of hydrogen peroxide *in statu nascendi* seems to be of value only in comparatively pure enzyme systems, in which stoichiometric oxygen consumption is secured.

SUMMARY

When histamine is oxidized by histaminase, both the amount of oxygen consumed and the rate of oxygen uptake vary greatly, depending upon the purity of the histaminase. The presence of hemin has been shown to increase oxygen uptake. Possibly still other impurities affect the uptake. Attempts to confirm the formation of H_2O_2 in the course of the histamine-histaminase reaction failed.

BIBLIOGRAPHY

1. Zeller, E. A., *Helv. chim. acta*, **21**, 880 (1938).
2. Zeller, E. A., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **2**, 93 (1942).
3. Kiese, M., *Biochem. Z.*, **305**, 22 (1940).
4. Zeller, E. A., *Helv. chim. acta*, **23**, 1418 (1940).
5. Keilin, D., and Hartree, E. F., *Proc. Roy. Soc. London, Series B*, **119**, 141 (1936).



THE OXIDATION OF *p*-CRESOL BY PEROXIDASE*

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The enzyme peroxidase has been found to catalyze the oxidation by hydrogen peroxide of a variety of substrates, especially phenols (1) and aromatic amines. Some of the oxidation products from hydroquinone (2), pyrogallol (3), *o*-phenylenediamine (4), guaiacol (4, 5), aniline (6), *p*-toluidine (7), mesidine (8), catechol and homocatechol (9) have been determined, and it is apparent from these reactions that the enzyme generally facilitates an oxidative condensation of the substrate.

p-Cresol has long been used as a substrate for the qualitative detection of peroxidase activity, a positive reaction being determined by the production of a white precipitate. This precipitate has been examined and three of its components isolated. They were found to be identical with the three compounds previously obtained by Pummerer *et al.* (10, 11) through the oxidation of *p*-cresol with potassium ferricyanide. One of these was identified as a tetrahydrodibenzofurane derivative corresponding to Compound I, and was probably formed by secondary ring closure of the intermediate quinol ether (Fig. 1); the second compound was identified as 2,2'-dihydroxy-5,5'-dimethylbiphenyl (II) by direct comparison with the synthetic compound; the third substance appeared to be a terphenyl derivative (III). This reaction may be formulated according to Fig. 1, though other compounds which have not been obtained in a crystalline form are undoubtedly present in the white precipitate.

EXPERIMENTAL

10 gm. of *p*-cresol were dissolved in 1000 cc. of 0.1 M phosphate buffer of pH 6.5. A solution of horseradish peroxidase (1) was added, and additional quantities of the enzyme were introduced as the reaction proceeded; the precipitate seemed to remove the enzyme from solution. Small portions of hydrogen peroxide were added gradually with continuous stirring, and the reaction appeared to be fairly complete after the addition of 1 molecular equivalent. When sufficient peroxidase was used, complete

* A part of this study was carried out while one of us (W. W. W.) was a Fellow in the Medical Sciences of the National Research Council, 1938-40. We are indebted to Professors H. T. Clarke and O. Wintersteiner of Columbia University and R. A. Peters of Oxford University for their aid during this period.

precipitation was effected within 15 to 30 minutes, and little or no *p*-cresol remained unchanged.

The precipitate was centrifuged, washed with water, and sucked dry on a filter. It was dissolved in ether, filtered if necessary, and the ether solution was extracted alternately with 5 per cent NaOH and water to remove the phenolic fraction. The neutral residue obtained upon distillation of the ether was recrystallized from methyl alcohol to yield 1.34 gm. of Compound I; m.p. 122–123°.

The other two compounds were isolated from the phenolic fraction. The alkaline solution was acidified and extracted with ether. After removal of the ether, the residue was steam-distilled to remove small amounts of unchanged *p*-cresol. The non-volatile residue was subjected to fractionation under a high vacuum; $p = 1 \times 10^{-3}$ mm. of Hg. The

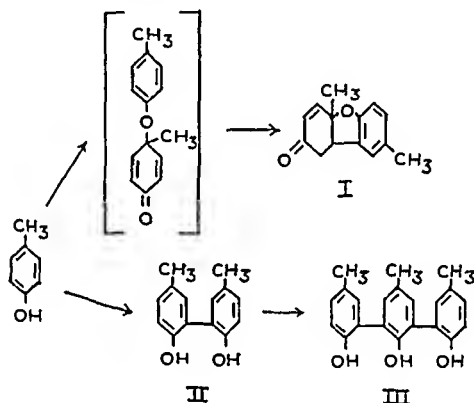


FIG. 1. The oxidation of *p*-cresol by peroxidase

most volatile fraction, collected between 150–180°, deposited crystals on long standing. Recrystallization from benzene and then dilute alcohol gave Compound II; m.p. 153–153.5°; yield 235 mg. Compound III was obtained from the fraction collected at 180–220°; crystals were readily obtained by dissolving the oily fraction in ether and precipitating with several volumes of petroleum ether in the cold. It was recrystallized from benzene; m.p. 196.5°; yield 685 mg.

Identification of Compound I—Compound I was a ketone, insoluble in NaOH; it gave no ferric chloride reaction, a positive Millon test, and coupled with diazotized *p*-nitroaniline to give an orange-red color. No acetyl derivative could be obtained. A 2 per cent solution in 95 per cent alcohol had no optical activity. The ultraviolet absorption spectrum (Fig. 2) showed a maximum at 301 m μ , molecular extinction coefficient = 3100.

It was identical with the ketone prepared from *p*-cresol by the ferri-

cyanide oxidation method of Pummerer *et al.* (10), and showed no depression of melting point on mixing. The oxime and semicarbazone were likewise identical. On treatment with HBr the ketone formed the biphenyl derivative identified by Pummerer *et al.* (11) as 2,3'-dihydroxy-5,6'-dimethylbiphenyl (IV). Methylation of this product with dimethyl sulfate and oxidation with KMnO_4 in alkali gave the dicarboxylic acid (V).

This ketone was assigned the tetrahydrodibenzofurane structure (I) by Pummerer *et al.* (11). Supporting evidence for this structure has been obtained by oxidative degradation of the ketone. A dicarboxylic acid

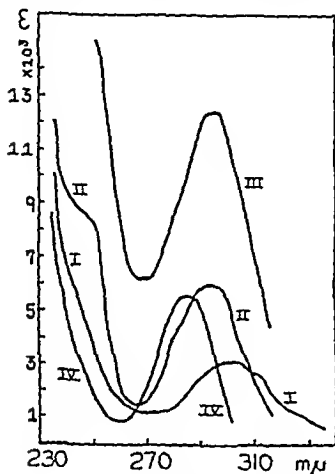


Fig. 2

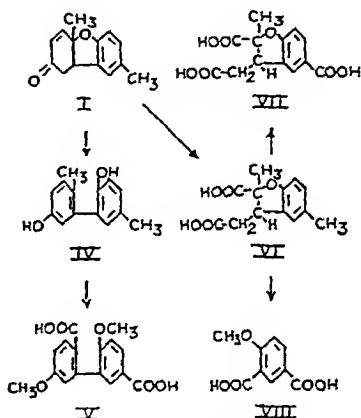


Fig. 3

Fig. 2. The ultraviolet absorption spectra of Compounds I, II, III, and IV in 95 per cent alcohol, plotted as the molecular extinction coefficient (ϵ) against wavelength ($m\mu$).

Fig. 3. Oxidative degradation of Compound I.

(VI) was obtained from the ketone on treatment with KMnO_4 in acetone. Further oxidation of this acid with KMnO_4 in alkali gave the tricarboxylic acid (VII) without additional loss of carbon atoms. Fusion of the dicarboxylic acid (VI) with KOH , methylation of the liberated phenolic group, and complete oxidation of the side chains with KMnO_4 in alkali gave 4-methoxyisophthalic acid (VIII). These reactions are illustrated in Fig. 3, and in conjunction with the evidence presented by Pummerer *et al.* (10, 11) leave little doubt of the original structure.

Analysis of Compound I— $\text{C}_{14}\text{H}_{14}\text{O}_2$

Calculated. C 78.50, H 6.54, mol. wt. 214

Found. " 78.18, 78.35, 77.98, H 6.57, 6.22, 6.38, mol. wt. (Rast) 216

Oxime—35 mg. of the ketone dissolved in 3 cc. of alcohol were treated at room temperature or under a reflux with 60 mg. of hydroxylamine hydrochloride and 70 mg. of sodium acetate dissolved in 2 cc. of water. The precipitated oxime was centrifuged, washed with hot water, and recrystallized from 2 cc. of hot 95 per cent alcohol; m.p. 198–200°.

Analysis— $C_{14}H_{15}O_2N$. Calculated, N 6.11; found, N 5.99

Semicarbazone—20 mg. of the ketone dissolved in 3 cc. of alcohol were treated for 24 hours at room temperature with 0.5 cc. of a 90 per cent alcoholic solution containing 50 mg. of semicarbazide acetate. The crystalline precipitate was washed thoroughly with hot water and alcohol; m.p. 255–256°. Recrystallization from glacial acetic acid did not alter the melting point.

HBr Treatment—100 mg. of the ketone were treated with 5 cc. of 48 per cent aqueous HBr on the steam bath for 1 hour. The product was extracted with ether, washed with $NaHCO_3$, and the phenols removed with 10 per cent NaOH. After acidification and extraction with ether this gave 96 mg. of a semicrystalline product, of which 85 mg. were recovered in pure form by sublimation under a high vacuum or in smaller amounts by recrystallization from ether-petroleum ether or benzene, and finally from dilute methyl alcohol to give Compound IV; m.p. 157.5–158.5°. The same product was obtained in smaller yields by treating the ketone with aqueous HI, or by refluxing it with alcoholic NaOH. The crystals gave a positive Millon test, and no semicarbazone. The ultraviolet absorption spectrum (Fig. 2) showed a maximum at 286 m μ , molecular extinction coefficient = 5500.

Analysis— $C_{14}H_{14}O_2$. Calculated. C 78.50, H 6.54
Found. " 77.81, " 6.30

Dibenzoate—30 mg. of Compound IV dissolved in 4 cc. of 10 per cent NaOH were shaken with 0.2 cc. of benzoyl chloride. The precipitate was filtered, washed with water, and recrystallized from dilute ethyl alcohol; yield 46 mg.; m.p. 131.5–132°.

Analysis— $C_{25}H_{22}O_4$. Calculated. C 79.62, H 5.21
Found. " 79.56, " 5.09

Methylation and Oxidation of Compound IV—0.5 gm. of Compound IV was methylated with dimethyl sulfate in 10 per cent NaOH. The ether-soluble residue was refluxed 7½ hours with 4 gm. of $KMnO_4$ in 100 cc. of 1 per cent NaOH. After the MnO_2 was filtered off, the filtrate was acidi-

fied, and the precipitated acid (V) was recrystallized from glacial acetic acid; yield approximately 200 mg.; m.p. 263–264°.

Analysis— $C_{14}H_{14}O_4$. Calculated. C 63.57, H 4.64
Found. " 62.83, " 4.87

Oxidation of Compound I—5.0 gm. of the ketone were dissolved in 200 cc. of acetone and cooled with a cold water bath, while 13 gm. of powdered $KMnO_4$ were added over a period of 1 hour with continuous stirring. The precipitate was filtered, washed with acetone, and then leached with hot water and 1 per cent NaOH. The aqueous leachings were acidified and extracted with ether; the ether-soluble residue was crystallized directly from benzene to give 2.3 gm. of the crude acid (VI). Recrystallization from ether-benzene, and finally from very dilute methyl alcohol, gave the pure acid; m.p. 149–150°.

Analysis— $C_{12}H_{14}O_5$
Calculated. C 62.40, H 5.60
Found. " 61.99, 62.13, H 5.58, 5.73

21.8 mg. required 17.26 cc. of 0.01 N NaOH for neutralization to phenolphthalein; neutralization equivalent 126, theoretical 125. On treatment with acetic anhydride in pyridine, this acid formed an anhydride, m.p. 125–126°.

Oxidation of Acid (VI)—300 mg. of the acid (VI) were dissolved in 25 cc. of 0.5 N NaOH and treated at room temperature with 550 mg. of $KMnO_4$ in 25 cc. of water. When this had decolorized (24 to 48 hours), the MnO_2 was filtered off, and the filtrate acidified and cooled. The crystals of acid (VII) were collected and recrystallized from very dilute methyl alcohol; yield 270 mg.; m.p. 238–240°.

Analysis— $C_{13}H_{12}O_7$
Calculated. C 55.71, H 4.29
Found. " 55.68, 55.57, H 4.37, 4.49

21.0 mg. required 22.28 cc. of 0.01 N NaOH for neutralization; neutralization equivalent 94.2, theoretical 93.3.

KOH Fusion of Acid (VI), Methylation, and Oxidation—2.0 gm. of the acid (VI) plus 8.0 gm. of KOH were fused at approximately 250° for 1 hour. On cooling, the melt was dissolved in water, acidified, and extracted with ether. The ether was washed with 5 per cent Na_2CO_3 and distilled to give a phenolic residue, which was dissolved in 10 cc. of 10 per cent NaOH and methylated with 2 cc. of dimethyl sulfate. Extraction of the methylated phenol with ether, washing with 10 per cent NaOH, and distillation of the ether gave an oily residue that was refluxed for 6 hours with 4 gm. of $KMnO_4$ in 100 cc. of 2 per cent NaOH. The filtrate, obtained

after decomposition of the excess KMnO_4 with alcohol and removal of the MnO_2 , was acidified and chilled to give 193 mg. of crystalline 4-methoxyisophthalic acid (VIII). Recrystallization from dilute methyl alcohol gave a pure product, m.p. $261\text{--}262^\circ$, that showed no depression of melting point when mixed with a sample of 4-methoxyisophthalic acid prepared from *m*-xyleneol.

Analysis— $\text{C}_9\text{H}_8\text{O}_5$. Calculated. C 55.10, H 4.08
Found. " 55.44 " 4.68

20.8 mg. required 20.66 cc. of 0.01 *N* NaOH for neutralization; neutralization equivalent 100, theoretical 98.

Refluxing the methoxy acid (VIII) for 3 hours with a mixture of 3 parts of 47 per cent aqueous HI and 1 part of glacial acetic acid, and recrystallization from hot water gave 4-hydroxyisophthalic acid; m.p. $305\text{--}306^\circ$. No depression of melting point was obtained when it was mixed with a synthetic sample. Dilute solutions gave a cherry-red ferric chloride reaction.

Identification of Compound II—Compound II was identical with 2,2'-dihydroxy-5,5'-dimethylbiphenyl (11, 12). A mixed melting point with the synthetic compound showed no depression. The ultraviolet absorption spectrum (Fig. 2) showed a maximum at 295 $\text{m}\mu$, molecular extinction coefficient = 6000.

Analysis— $\text{C}_{14}\text{H}_{14}\text{O}_2$
Calculated. C 78.50, H 6.54, mol. wt. 214
Found. " 78.39, " 6.93 " " (Rast) 227

Diacetate—15 mg. of Compound II were dissolved in 1 cc. of pyridine and 0.5 cc. of acetic anhydride, and allowed to stand at room temperature 24 hours. The precipitate obtained upon dilution with ice water was centrifuged and recrystallized from dilute methyl alcohol; m.p. 88° .

Analysis— $\text{C}_{18}\text{H}_{18}\text{O}_4$. Calculated. C 72.48, H 6.04
Found. " 72.47, " 5.91

Synthesis of 2,2'-Dihydroxy-5,5'-dimethylbiphenyl—25.8 gm. of 3-bromo-4-methoxytoluene in 50 cc. of dry ether were treated with 3.2 gm. of magnesium. After the magnesium had dissolved, 20 gm. of anhydrous cuprous chloride suspended in 50 cc. of ether were added, and the solution refluxed 5 hours. Ice and 1:1 HCl were then added to dissolve the copper salt. The ether layer and ether extracts of the aqueous layer were combined, washed with 1:3 NH_4OH , dilute HCl, and water. The oily residue obtained from distillation of the ether was refluxed 3 hours with a mixture

of 1 part of glacial acetic acid and 2 parts of 47 per cent aqueous HI. The reaction mixture was diluted with water, extracted with ether, and the ether washed thoroughly with Na_2CO_3 . The phenols were removed from the ether by extraction with 10 per cent NaOH, and recovered with ether after acidification of the NaOH extracts. The yield after recrystallization from benzene was 1.42 gm.; m.p. 153° .

Identification of Compound III—This compound was apparently identical with one obtained by Pummerer *et al.* (10, 11). It was soluble in NaOH, and gave a negative Millon test and a greenish ferric chloride reaction in dilute alcohol. It formed no semicarbazone. The ultraviolet absorption spectrum (Fig. 2) showed a maximum at $295\text{ m}\mu$, molecular extinction coefficient = 12,400.

Analysis— $\text{C}_{21}\text{H}_{20}\text{O}_2$

Calculated. C 78.75, H 6.25, mol. wt. 320

Found. " 78.53, " 6.37, " " (Rast) 296

Triacetate—Acetylation in pyridine with acetic anhydride at room temperature by the usual procedure gave an oil that crystallized on standing; m.p. 107° .

Analysis— $\text{C}_{27}\text{H}_{24}\text{O}_4$. Calculated. C 72.65, H 5.83

Found. " 72.41, " 5.84

Benzoate—A benzoate was prepared by the Schotten-Baumann method and crystallized from methyl alcohol with benzene to aid in dissolving the derivative. The crystals were insoluble in NaOH; m.p. $189\text{--}190^\circ$.

Pummerer *et al.* first (10) believed this compound to be a biphenyl derivative of the formula $\text{C}_{14}\text{H}_{14}\text{O}_2$, but later (11) could not identify it with any of the possible isomers. Since all of the analyses agree equally well with a biphenyl or terphenyl structure, differentiation between the two possibilities is based entirely on the molecular weight determinations. By analogy with Compound II, this substance is believed to possess the terphenyl structure (III).

DISCUSSION

Johnson and Tewkesbury (13) recently postulated a mechanism for the formation of thyroxine from diiodotyrosine; this mechanism is analogous to the type of oxidation herein obtained with *p*-cresol in the formation of Compound I, and was based on the previously cited results obtained by Pummerer *et al.* (10, 11) in their study of the oxidation of *p*-cresol by potassium ferricyanide. If diiodotyrosine were oxidized in a manner analogous to the oxidation of *p*-cresol, the quinol ether first produced would be intermediate in the formation of thyroxine, requiring only the removal of

one alanine side chain to complete the synthesis. The presence of the 2 iodine atoms ortho to the phenolic hydroxyl would block the formation of biphenyl and terphenyl derivatives, and would also block the secondary ring closure of the quinol ether to a dibenzofurane derivative. The observation, reported in this paper, that this type of oxidation is brought about by the enzyme peroxidase suggests that this synthesis may be brought about by biological means.

SUMMARY

Three compounds were isolated from the white precipitate produced by the oxidation of *p*-cresol with peroxidase and hydrogen peroxide. They were found to be identical with the three compounds previously obtained by Pummerer *et al.* from the oxidation of *p*-cresol with potassium ferricyanide. One was identified as a tetrahydrodibenzofurane derivative, the second was found to be 2,2'-dihydroxy-5,5'-dimethylbiphenyl, and the third was believed to be an analogous terphenyl derivative.

We are indebted to Mr. William Saschek of Columbia University and to Dr. Y. Subbarow of the Lederle Laboratories, Inc., for the elementary analyses.

BIBLIOGRAPHY

1. Elliott, K. A. C., *Biochem. J.*, **26**, 1281 (1932).
2. Raper, H. S., *Physiol. Rev.*, **8**, 245 (1928).
3. Willstätter, R., and Heiss, H., *Ann. Chem.*, **433**, 17 (1923).
4. Chodat, R., in Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Berlin and Vienna, Abt. IV, Teil 1, 319 (1920).
5. Bertrand, G., *Compt. rend. Acad.*, **134**, 124 (1903).
6. Mann, P. J. G., and Saunders, B. C., *Proc. Roy. Soc. London, Series B*, **119**, 47 (1935-36).
7. Saunders, B. C., and Mann, P. J. G., *J. Chem. Soc.*, 769 (1940).
8. Chapman, N. B., and Saunders, B. C., *J. Chem. Soc.*, 496 (1941).
9. Pugh, C. E. M., and Raper, H. S., *Biochem. J.*, **21**, 1370 (1927).
10. Pummerer, R., Melamed, D., and Puttfarhen, H., *Ber. chem. Ges.*, **55**, 3116 (1922).
11. Pummerer, R., Puttfarhen, H., and Schopfloeher, P., *Ber. chem. Ges.*, **58**, 1808 (1925).
12. Fichter, F., and Aekermann, F., *Helv. chim. Acta*, **2**, 583 (1919).
13. Johnson, T. B., and Tewkesbury, L. B., Jr., *Proc. Nat. acad. Sc.*, **28**, 73 (1942).

THE EFFECT OF pH ON THE FORMATION OF PYRROLIDONE-CARBOXYLIC ACID AND GLUTAMIC ACID DURING ENZYMATIC HYDROLYSIS OF GLUTATHIONE BY RAT KIDNEY EXTRACT

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(Received for publication, July 31, 1942)

Aqueous extracts of rat kidney were shown previously (1) to bring about complete enzymatic hydrolysis of glutathione (GSH), as indicated by the nearly quantitative liberation of cysteine. The accompanying increase in carboxyl groups indicated the presence of the three constituent amino acids, cysteine, glycine, and glutamic acid, in almost theoretical amounts. The present study on the effect of pH upon this hydrolysis shows that a part of the glutamic acid may be split off as the anhydride, pyrrolidone-carboxylic acid, the proportion of these two compounds depending upon the pH of the digestion mixture.

Methods

1:5 kidney extracts were prepared from pooled fresh kidneys of normal albino rats, by grinding with sand, extracting with 5 volumes of water, centrifuging, and decanting the supernatant extract. Incubation of GSH with the kidney extract was carried out at 25° and the pH of the mixtures was measured with the Beckman (glass electrode) pH meter. COOH determinations were made by titrating 1 cc. of the digestion mixtures with 0.05 N NaOH according to the alcohol-formaldehyde method of Harris (2).

Cysteine and GSH Determination—The digestion mixtures were deproteinized by the addition of 11 volumes of 2.2 per cent sulfosalicylic acid. The acid filtrates were submitted to electrolytic reduction (3) before being used for cysteine or GSH estimations, in order to reduce any cystine or GSSG which may have been formed during the digestion.

Cysteine was estimated by means of the Sullivan reaction (4), the colors being read through a green filter (Wratten No. 62). Cysteinylglycine, if present, would interfere with this determination, since it also gives a red color, which, however, contains more purple (5) than that produced by cysteine. The colors obtained from the protein-free filtrates indicated that very little, if any, cysteinylglycine could be present, except possibly in filtrates from digestion mixtures of pH of about 8 and higher. In the latter case a slightly purplish shade was obtained. Control determinations

of cysteine in the presence of amounts of GSH which could be present in the filtrates showed that GSH did not introduce any error into these determinations.

GSH was estimated by means of the manometric glyoxalase method (6).

Glutamic Acid Determination—The glutamic acid content of filtrates of the digestion mixtures deproteinized by the use of tungstic acid was determined by the succinoxidase method of Cohen (7), with rat heart as the source of this enzyme. Control determinations of glutamic acid in the presence of cysteine, glycine, and pyrrolidonecarboxylic acid showed that no interference occurred and that 93 to 96 per cent recovery of glutamic acid was obtained.

Pyrrolidonecarboxylic Acid Determination—Pyrrolidonecarboxylic acid was determined by a modification of the method described by Pucher and Vickery (8). The method is based upon the solubility of pyrrolidonecarboxylic acid in ethyl acetate and its conversion to glutamic acid by acid hydrolysis, the resulting increase in amino nitrogen being a measure of the pyrrolidonecarboxylic acid.

A protein-free filtrate was prepared from the digestion mixture by addition of 1 volume of water, $\frac{2}{3}$ volume of $\frac{2}{3}$ N H_2SO_4 , and $\frac{1}{3}$ volume of 10 per cent Na_2WO_4 . A volume of filtrate containing preferably 5 to 10 mg. of pyrrolidonecarboxylic acid was introduced into a continuous ether extractor (capacity about 25 cc.) with 10 cc. of 0.1 M phosphate buffer (pH 7.4) and adjusted to pH 2.4 to 2.6 with H_2SO_4 . The mixture then was diluted up to the capacity of the extractor with 20 per cent Na_2SO_4 . After extraction with freshly distilled ethyl acetate for 20 hours, during which the aqueous solution was maintained at 20–25°, the solvent was removed from the extract by evaporation at 100°, and the residue taken up in a known volume of water and filtered. In order to convert pyrrolidonecarboxylic acid to glutamic acid, an aliquot was made 2 N with respect to HCl, heated for 2 hours at 100°, adjusted to pH 4 to 5, and diluted to a known volume. Similar solutions were prepared from the enzyme blank.

The free amino acid contents of the hydrolyzed and unhydrolyzed extracts were then determined from the CO_2 evolved on treatment with chloramine-T at pH 4.7, the Barcroft-Warburg technique described by Cohen (7) for glutamic acid being used. Pyrrolidonecarboxylic acid was found to give no CO_2 under these conditions, while glutamic acid yielded 1 mole of CO_2 . Known amounts of pyrrolidonecarboxylic acid after acid hydrolysis gave CO_2 values indicating that 97 to 98 per cent conversion to glutamic acid had occurred. The increase in CO_2 production upon hydrolysis of the extract, therefore, was used as a measure of the pyrrolidonecarboxylic acid present.

Control determinations made on solutions of pyrrolidonecarboxylic acid

alone and in the presence of glycine, glutamic acid, and cysteine, with extraction times of 15 to 20 hours, showed recovery values of 93 to 95 per cent. Negligible CO_2 values for the unhydrolyzed extracts indicated that the amino acids were not extracted.

EXPERIMENTAL

pH Optimum for Hydrolysis—To obtain digestion mixtures of pH values from 5 to 9.5, varied amounts of from 0.12 to 0.4 cc. of 1.0 N NaOH were added to 1 cc. portions of 0.16 M GSH, enough water added to make 2 cc. of 0.08 M GSH, and then 2 cc. of 1:5 kidney extract added. The resulting digestion mixtures were all 0.04 M with respect to GSH and 1:10 with respect to kidney. GSH, cysteine, and COOH determinations were made after a digestion period of 30 minutes. No blank kidney mixtures were made, since it was known from previous experience that the increase in the blank due to autolysis would be insignificant for this short period. The maximum change in pH of any mixture during the digestion period was 0.1 pH unit.

The effect of pH on the loss of GSH and on the formation of cysteine and COOH groups is shown in Fig. 1. The data are expressed in terms of the total change theoretically possible; that is, cysteine is calculated on a basis of 1 mole from each mole of GSH used, and COOH on a basis of 2 equivalents which could be liberated if the two peptide bonds of GSH were split.

The observed pH optimum was found to depend upon the method used to follow the hydrolysis. When GSH loss was measured, the pH optimum was about 8.5; for cysteine formation, about 7.8; and for COOH liberation, about 7.0. Since GSH was estimated by the glyoxalase method, which requires the intact tripeptide,¹ its loss could have been due to the splitting of only one peptide bond with formation of either possible dipeptide, cysteinylglycine or γ -glutamylcysteine, but the formation of cysteine requires the splitting of both peptide bonds. It is readily understandable, therefore, why the cysteine curve should fall below the GSH loss curve, but it is not at once apparent why the COOH curve should fall below the cysteine curve. In fact at pH 7 and higher, the COOH liberation was far below that to be expected from the amount of cysteine formed. In order to investigate the reason for this discrepancy, the following experiments were carried out.

Time Curves for Hydrolysis at pH 7.6, 6.9, and 5.2—Digestion mixtures

¹ It was found several years ago (unpublished data by one of us, G. E. W.) that neither cysteinylglycine nor γ -glutamylcysteine could activate glyoxalase. The cysteinylglycine was prepared in solution by heating GSH at 62° for 5 days according to Mason's procedure (5). The glutamylcysteine was a small synthetic sample kindly supplied by Dr. C. R. Harington (9).

were made containing 0.04 M GSH and 1:10 kidney extract. The mixture at pH 7.6 contained no added buffer, being adjusted to this pH by NaOH. The mixture at pH 6.9 contained 0.01 M phosphate buffer, and that at pH 5.2 contained 0.025 M acetate buffer. Blank 1:10 kidney extracts of corresponding pH were also made. Samples were removed at various time intervals for COOH, GSH, and cysteine determinations.

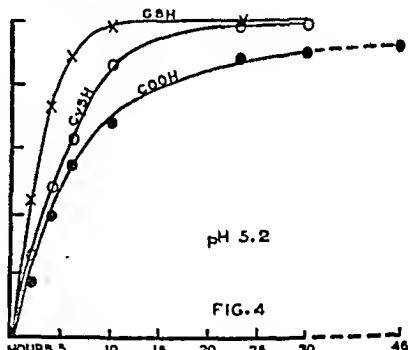
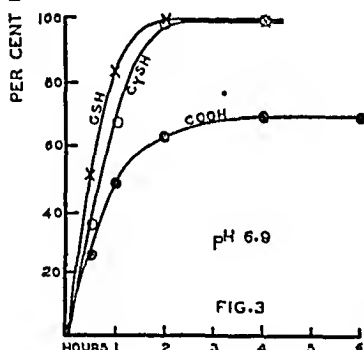
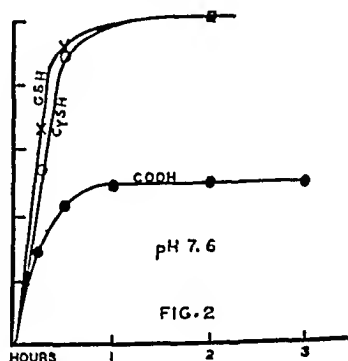
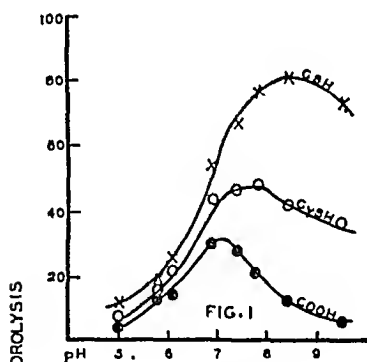


FIG. 1. pH optimum for hydrolysis of 0.04 M GSH by 1:10 kidney extract, as indicated by GSH loss, cysteine (CySH) formation, and COOH increase, during 30 minutes digestion at 25°.

FIGS. 2 TO 4. Hydrolysis of 0.04 M GSH by 1:10 kidney extract at 25° and various pH values, as indicated by GSH loss, cysteine (CySH) formation, and COOH increase.

The data obtained are given in Figs. 2 to 4. As the time of hydrolysis was prolonged, the GSH was completely destroyed and eventually an equivalent amount of cysteine appeared, showing that both peptide bonds of GSH had been broken. In the early stages of the hydrolysis, however, an equivalent amount of cysteine was not present, indicating the presence of a dipeptide as an intermediate in the breakdown. For every mole of

cysteine formed 2 equivalents of COOH should be liberated, and for every mole of dipeptide 1 equivalent should appear. It would be expected, therefore, that the curve for COOH formation would fall between the curves for cysteine formation and for GSH loss. Since the COOH curve always fell below the cysteine curve, there was indication that a part of the glutamic acid or glycine was present in a form which did not contain the free COOH group expected. The final COOH value, which reached 51 per cent at pH 7.6, 70 per cent at pH 6.9, and 90 per cent at pH 5.2, indicated more or less of this product, depending upon the pH of the digestion mixture.

The reason for the low COOH formation became apparent when a glutamic acid analysis was carried out on the completely hydrolyzed mixture at pH 6.9. The data in Table I show that the amount present was only 47 per cent of the theoretical, and that the COOH increase calculated from this figure (73.5 per cent) was in good agreement with the observed value (70 per cent). The remainder of the glutamic acid, therefore, must have been split off in a form which did not contribute to the COOH increase. Since its presence in the anhydride form, pyrrolidonecarboxylic acid, was the logical assumption, the tungstic acid filtrate of the digestion mixture was hydrolyzed 2 hours at 100° in 2 N HCl, conditions known to open the pyrrolidone ring. The glutamic acid value was increased thereby to 93 per cent of the theoretical. Additional evidence that the increase was due to pyrrolidonecarboxylic acid was obtained in later experiments.

Direct analysis for pyrrolidonecarboxylic acid (Table I) in the digestion mixture at pH 7.6 at the end of hydrolysis gave a value equivalent to 87 per cent of the glutamic acid content of the original GSH. Confirmation that the increase in free amino acid upon acid hydrolysis of the material extracted by ethyl acetate was due to conversion of pyrrolidonecarboxylic acid to glutamic acid was obtained by analysis for the latter after the acid hydrolysis. The amount found accounted for 98 per cent of the free amino acid increase. Assuming that the other 13 per cent of the possible glutamic acid was free in the digestion mixture at the end of hydrolysis, the theoretical COOH increase would be 56.5 per cent. A value of 51 per cent was found.

Analysis of the mixture at pH 5.2 (Table I) gave values of 69 per cent for glutamic acid and 20 per cent for pyrrolidonecarboxylic acid, which likewise accounted very well for the observed COOH increase.

The foregoing results show that the COOH increase is not a true measure of the extent of hydrolysis, as might be expected. However, on the theoretical basis of the equations given below Table I, the value obtained at complete hydrolysis can be used to calculate the amounts of glutamic and pyrrolidonecarboxylic acids present.

Relation between pH and Formation of Glutamic and Pyrrolidonecarboxylic Acids—The experiments which showed that pyrrolidonecarboxylic acid predominated at a slightly alkaline pH and glutamic acid at an acid pH had

TABLE I

Glutamic Acid and Pyrrolidonecarboxylic Acid Analysis of GSH-Kidney Digestion Mixtures after Complete Hydrolysis at 25°

	Digestion at pH 5.2, 48 hrs		Digestion at pH 6.9, 5 hrs		Digestion at pH 7.6, 3 hrs	
	Digest	Blank	Digest	Blank	Digest	Blank
Glutamic acid						
GSH originally present, <i>mg</i>	8	19		8	19	
Glutamic acid equivalent of original GSH, <i>mg</i> .	3	92		3	92	
Total O ₂ absorbed, <i>microliters</i>	232		27	165		26
Net O ₂ absorbed, <i>microliters</i>	205			139		
Glutamic acid found,* <i>mg</i>	2	69		1	83	
" " " %	69			47		
COOH increase based on glutamic acid found,† %	84			73	5	
COOH increase found by titration, %	90			70		51
Pyrrolidonecarboxylic acid						
GSH originally present, <i>mg</i>	19	24				20
Pyrrolidonecarboxylic acid equivalent of original GSH, <i>mg</i>	8	08				8
CO ₂ evolved from EtOAc extract						
After hydrolysis, <i>microliters</i>	411		77		1305	2
Before " "	70		14		6	0
Net, <i>microliters</i>	278				1297	
Pyrrolidonecarboxylic acid found,† <i>mg</i>	1	60			7	48
" " " %	20			46§	87	
COOH increase based on pyrrolidonecar- boxylic acid found,† %	90			77		56

* 1 mg of glutamic acid is equivalent to 76.12 microliters of O₂.

† Calculated according to the equations, per cent COOH increase = $\frac{1}{2}$ (100 + per cent glutamic acid), per cent COOH increase = $\frac{1}{2}$ (200 minus per cent pyrrolidonecarboxylic acid). The COOH increase is based on a theory of 2 equivalents of COOH per mole of GSH.

† 1 mg of pyrrolidonecarboxylic acid is equivalent to 173.5 microliters of CO₂.

§ Based on the increase in the glutamic acid content found after hydrolysis of the protein-free digest with 2 N HCl for 2 hours at 100°

been carried out with different preparations of kidney extract. In order to obtain curves (Fig. 5) showing the proportion of these two compounds formed at a number of pH values, with the same kidney extract, digestion

mixtures were made containing 0.02 M GSH and 1:10 kidney extract. The mixture at pH 4.6 was buffered with 0.025 M acetate and the other mixtures with 0.01 M phosphate. Blank kidney extracts were buffered in the same manner. The hydrolysis was carried to the maximum COOH increase and the pyrrolidonecarboxylic acid and glutamic acid formation calculated from this according to the equations given below Table I, which have been shown to be valid in this pH range.

With this kidney extract, glutamic acid predominated below pH 6.6; in the region of pH 6.3 there was a rather sharp increase in the proportion of pyrrolidonecarboxylic acid; at pH 7.5 almost all of the glutamic acid was in the form of pyrrolidonecarboxylic acid. In general, other kidney extracts gave similar results, deviations, if any, being most noticeable in the pH range 6 to 7, where small changes in pH produced the greatest effect.

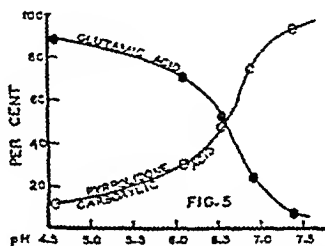


FIG. 5. Effect of pH on glutamic acid and pyrrolidonecarboxylic acid formation during complete hydrolysis of 0.02 M GSH by 1:10 kidney extract at 25°, as calculated from maximum COOH increase.

Since this effect of pH was not known at the time of the original work (1), the pH of the digestion mixtures was not carefully controlled, kidney extract having been mixed with GSH solutions to which 1 equivalent of NaOH had been added. The 90 per cent COOH increase obtained at that time would indicate, on the basis of the present work, that probably the pH had been in the range 6 to 6.5 and that 20 per cent of pyrrolidonecarboxylic acid had been present.

Isolation of Pyrrolidonecarboxylic Acid—In order to prove conclusively that the glutamic acid derivative extracted by ethyl acetate during the analytical procedure was pyrrolidonecarboxylic acid and not another anhydride of glutamic acid, isolation was undertaken. A duplicate of the previously described digestion mixture at pH 7.6 was made, since this pH was favorable for maximum formation of the anhydride. After 2.5 hours of digestion the COOH increase had become constant at 53.5 per cent, which indicated that 93 per cent of the glutamic acid was in the anhydride form. The mixture was deproteinized with tungstic acid, and 50 cc. of the filtrate, equivalent to 205 mg. of GSH, were adjusted to pH 2.4 with

6 N NaOH. Half of the solution was then put into each of two 25 cc. continuous extractors. Extraction was carried out with freshly distilled ethyl acetate for 10 hours, during which the aqueous solutions were kept at 20°. The combined extracts were evaporated to dryness at 100°. The residue was dried *in vacuo* over H₂SO₄, and then extracted with four 2 cc. portions of boiling ethyl acetate. After evaporation of these extracts to a small volume, 30 mg. of a crystalline product separated on cooling; m.p. 153–157°. Recrystallization from ethyl acetate raised the melting point to 160°. A known sample of *l*(–)-pyrrolidonecarboxylic acid, prepared from *l*(+)-glutamic acid (10), and recrystallized from ethyl acetate, melted at 159–160°; this value was not lowered by admixture with the isolated material. The latter contained 10.82 per cent N (micro-Kjeldahl); theoretical value for C₅H₇O₃N, 10.85 per cent. As with the known acid, no CO₂ was evolved on treatment with chloramine-T; after hydrolysis with 2 N HCl for 2 hours at 100°, 1 mg. yielded 166 microliters; theoretical value 173.5 microliters.

Incubation of Glutamic Acid and Pyrrolidonecarboxylic Acid with Kidney Extract—The possibility that glutamic acid first was formed during hydrolysis of GSH and then converted into pyrrolidonecarboxylic acid was eliminated by studies on the stability of glutamic acid in kidney extract under the conditions of the hydrolysis experiments. When 0.04 M *l*(+)-glutamic acid was incubated with 1:15 kidney extract at pH 8, 7, or 5.4 for 22 hours, no ring closure occurred, as indicated by a COOH titration which was constant when corrected for the increase found in corresponding blanks. Neither was there any ring closure when 0.04 M *l*(–)-cysteine, or cysteine plus glycine, was incorporated as a possible activator at pH 7.0 or 7.5. Ring closure must have occurred, therefore, before or at the moment of splitting of the γ -glutamyl peptide bond.

The results of similar studies to show whether pyrrolidonecarboxylic acid could be converted to glutamic acid by ring opening were also negative. No increase in COOH was observed when 0.04 M *l*(–)-pyrrolidonecarboxylic acid, with or without added cysteine or cysteine plus glycine, was incubated with 1:15 kidney extract at pH 5.4 or 7.0. Moreover, when a digestion mixture of GSH and kidney extract at pH 7.1, showing a maximum COOH increase of 58 per cent, was adjusted to pH 6.1, no further increase in COOH could be obtained. These experiments all indicate that pyrrolidonecarboxylic acid and glutamic acid were formed directly at the time of splitting of the γ -glutamyl bond and not by any secondary reaction.

DISCUSSION

The two possible substrates for the formation of pyrrolidonecarboxylic acid are, of course, GSH and the dipeptide, γ -glutamylcysteine, which

would be present if glycine were split off first from GSH. Although formation of pyrrolidonecarboxylic acid by spontaneous splitting of the γ -glutamyl bond of GSH in aqueous solution had been observed by Mason (5), complete cleavage required 5 to 7 days at 62°, while at 37° the cleavage was only 80 per cent in 14 days. It would appear, therefore, that spontaneous decomposition of GSH could not account for the formation of pyrrolidonecarboxylic acid under the mild conditions of our experiments. γ -Glutamylcysteine also would not be expected to decompose spontaneously at 25° and pH 7.6 in 1 hour, since it had been synthesized and recovered from aqueous solution (9), and since no cysteine had been liberated (1) in 30 minutes under the alkaline conditions of the Sullivan reaction. Regardless of whether pyrrolidonecarboxylic acid is formed directly from GSH or from the dipeptide, its formation seems definitely to be due to the enzymatic action of the kidney extract. This, as far as we know, is the first reported evidence for the formation of pyrrolidonecarboxylic acid by enzymatic action.

The formation of different proportions of glutamic acid and pyrrolidonecarboxylic acid, depending upon the pH, suggests that perhaps two different enzymes with different pH optima are involved, or that each acid is formed from a different substrate, one from GSH and the other from γ -glutamylcysteine. A better understanding of the mechanism of the enzymatic hydrolysis of GSH will have to await a separation of the enzymes concerned or the availability for study of the two dipeptides involved, cysteinylglycine and γ -glutamylcysteine.

SUMMARY

Both peptide bonds in GSH were found to be split by the enzymatic action of rat kidney extract in the pH range 5 to 9.5, as indicated by the liberation of cysteine. The rate was fastest in the range of pH 7 to 8.

Splitting of the γ -glutamyl peptide bond resulted in the formation of different proportions of glutamic and pyrrolidonecarboxylic acids, depending upon the pH of the digestion mixture; glutamic acid predominated at pH values lower than about 6.6, and pyrrolidonecarboxylic acid in the more alkaline range.

The evidence indicated that pyrrolidonecarboxylic acid, as well as glutamic acid, was formed directly by the enzymatic action of the kidney extract on the γ -glutamyl bond.

BIBLIOGRAPHY

1. Schroeder, E. F., and Woodward, G. E., *J. Biol. Chem.*, **120**, 209 (1937).
2. Harris, L. J., *J. Biol. Chem.*, **84**, 296 (1929).
3. Dohan, J. S., and Woodward, G. E., *J. Biol. Chem.*, **129**, 393 (1939).
4. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1421 (1929).

5. Mason, H. L., *J. Biol. Chem.*, **90**, 25 (1931).
6. Woodward, G. E., *J. Biol. Chem.*, **109**, 1 (1935).
7. Cohen, P. P., *Biochem. J.*, **33**, 551 (1939).
8. Pueher, G. W., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, **12**, 27 (1940).
9. Harington, C. R., and Mead, T. H., *Biochem. J.*, **29**, 1602 (1935).
10. Abderhalden, E., *Handbuch der biologischen Arbeitsmethoden*, Berlin and Vienna, Abt. I, Teil 7, 166 (1923).

THE INFLUENCE OF INSULIN UPON GLYCOGEN STORAGE IN THE DIABETIC RAT

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The physiological action of insulin is not definitely known. Not long after its discovery, Macleod (1) was of the opinion that, while in the diabetic organism insulin administration leads to increased deposition of glycogen and increased oxidation of glucose, in the normal organism it causes carbohydrate storage not as glycogen but possibly as some substance related to hexose phosphate and has no significant influence upon glucose oxidation. The latter suggestions as to the nature of insulin action have long been forgotten and it is now generally assumed that insulin exerts its effect upon glucose metabolism by increasing glucose oxidation and glycogen deposition, particularly in the muscles (2). One recent reviewer (3) states that the most firmly established effect of insulin is "the withdrawal of sugar from the blood and its deposition as glycogen in the muscles." Certain considerations, however, indicate that such processes are inadequate to explain all the known facts about insulin. One of these is the finding (4) that the action of insulin on carbohydrate metabolism is predominantly one of promoting storage. The extent of the capacity for this must be much greater than can be accounted for by the reported increases in muscle glycogen. Cori and Cori (5) studied the effect of insulin on glucose disposal in normal rats, and Best, Hoet, and Marks (6) examined the same thing in liverless cats. In both cases most of the sugar was found to have been deposited as muscle glycogen. But the amount of glucose entering the circulation in these experiments was very small and not of much greater magnitude than would be used during the period of observation. The results would put them under the range of normal storage requirements. A study has therefore been made of the influence of insulin upon the storage of glucose as glycogen under conditions in which storage quantities of glucose were being dealt with. These conditions are attained in the diabetic rat receiving a high carbohydrate diet, in which the administration of insulin results in a marked reduction in the loss of sugar in the urine and the storage of this sugar (4).

Methods

Young male rats weighing between 100 and 150 gm. were depancreatized by Greeley's adaptation¹ of the method of Shapiro and Pincus (7). They were allowed to remain on the stock diet of dog pellets until well recovered from the operation and then placed on a high carbohydrate diet, the same as that used by Drury (4) except that the glucose was replaced by sucrose. Daily urine sugar determinations were carried out and total nitrogen was determined during the 3 days previous to sacrificing the animals. The urine volumes varied with the glucose excreted and reached remarkable figures (Table I).

Frequent 24 hour fasts were employed in an attempt to reduce the stored fat to a basal level but this effort proved unsuccessful. Weight lost in fasting could not be restored without the aid of insulin, but unless the rats were fasted their weight was maintained. When maximum glycosuria and minimum body weight were reached, the rats were paired on the basis of their original body weight, daily food consumption, sugar excretion, and the sugar retained. One of each pair received 20 units of regular insulin three times during the day. The diabetic rat on a high carbohydrate diet is very insulin-resistant and this first insulin treatment had relatively little influence upon sugar storage. A fast day was next imposed and then the insulin administration repeated during another day which ended the observations. The insulin was then effective. At the end of this day the rats were fasted for a couple of hours to empty the gastrointestinal tract and then sacrificed by the intraperitoneal injection of sodium pentobarbital. The livers and the rest of the body were placed in separate flasks of hot 30 per cent potassium hydroxide for the determination of glycogen (8). The usual methods were used for the determination of blood sugar (9) and urine sugar (10).

The results obtained in this manner are presented in Table I.

Results

The data show quite clearly that in the diabetic rat insulin increases the storage of sugar as glycogen in both the liver and muscles. The glycogen concentration increased 200 per cent in the liver and 275 per cent in the rest of the body, while the amount increased 290 and 275 per cent respectively. However, of the total amount of sugar stored under the influence of insulin only 24 per cent can be accounted for as glycogen. 15 per cent of the stored sugar is in the form of liver glycogen and 9 per cent as glycogen in other parts of the organism, chiefly in the muscles in all probability.

¹ Greeley, P. O., personal communication.

TABLE I

Influence of Insulin upon Total Sugar Storage, Sugar Storage As Glycogen, and Nitrogen Retained in Diabetic Rats

Rat No	Body weight*	Body surface	Carbohydrate as glucose per sq dm body surface per day			Body glycogen		Liver per sq dm body surface	Liver glycogen		Urine per sq dm body surface per day		Blood sugar	
			Ingested†	Excreted	Retained	Concentration	Per sq dm body surface		Concentration	Per sq dm body surface	Total N	Volume		
Controls														
	gm	sq dm	gm	gm	gm	per cent	gm	gm	per cent	gm	gm	cc	mg per cent	
1	242	4.4	5.9	2.2	3.7	0.07	0.04	3.1	4.57	0.14	0.11	22	395	
2	200	3.9	7.4	3.1	4.3	0.07	0.04	3.3	2.85	0.09		32	437	
3	196	3.8	6.6	4.8	1.8	0.10	0.05	3.2	3.82	0.12	0.19	58	472	
4	193	3.8	7.5	4.3	3.2	0.27	0.14	3.0	0.70	0.21	0.14	40	424	
5	188	3.7	7.2	3.4	3.8	0.23	0.12	2.8	2.67	0.14	0.16	32	588	
6	178	3.6	9.7	4.6	5.1	0.11	0.05	3.7	4.15	0.15	0.18	57	547	
7	172	3.5	7.4	5.0	2.4	0.11	0.05	5.1	1.10	0.06	0.22	53	611	
8	162	3.4	6.9	3.1	3.8	0.12	0.06	3.4	5.89	0.18	0.11	36	518	
9	153	3.3	6.0	2.9	3.1	0.24	0.11	2.6	4.73	0.12	0.12	20	345	
10	150	3.2	6.4	3.3	3.1	0.27	0.13	2.7	3.84	0.10	0.12	30	430	
11	118	2.7	11.9	6.3	5.6	0.19	0.09	2.7	2.61	0.07	0.29	68	650	
12	105	2.5	7.5	4.8	2.7	0.12	0.05	2.3	2.21	0.05	0.16	40	439	
Average	171	3.5	7.5	4.0	3.5	0.16	0.08	3.2	3.26	0.12	0.16	41	458	
Insulin treated														
1	244	4.4	7.0	0.9	6.1	0.47	0.26	3.9	8.60	0.33	0.14	9	436	
2	228	4.2	6.1	0.7	5.4	0.60	0.33	3.9	8.74	0.34	0.09	9	373	
3	216	4.1	6.0	0.6	5.4	0.38	0.20	3.1	4.53	0.24	0.08	5	251	
4	197	3.8	8.5	1.8	6.7	0.60	0.31	5.3	4.74	0.24	0.14	18	593	
5	196	3.8	7.6	1.2	6.4	0.83	0.42	4.5	7.94	0.36	0.13	13	476	
6	180	3.6	6.7	2.3	4.4	0.39	0.20	3.4	7.18	0.25	0.11	10	377	
7	172	3.5	7.9	2.0	5.9	0.77	0.38	6.4	10.95	0.70	0.13	19	518	
8	172	3.5	8.7	2.0	6.7	0.70	0.35	5.5	14.17	0.79	0.14	23		
9	172	3.5	7.1	1.4	5.7	0.51	0.25	5.0	11.95	0.61	0.13	14	437	
10	170	3.5	7.6	2.0	5.6	0.77	0.35	4.9	12.10	0.59	0.12	20	398	
11	152	3.2	8.0	2.7	5.3	0.53	0.25	4.8	13.08	0.62	0.14	22	385	
12	124	2.8	10.0	3.3	6.7	0.69	0.30	4.4	13.28	0.58	0.17	23	337	
Average	185	3.7	7.6	1.7	5.9	0.60	0.30	4.6	9.78	0.47	0.12	15	416	

* When sacrificed

† Carbohydrate ingested is calculated in terms of glucose from total sucrose and carbohydrate from vitab or 92.55 per cent of the total diet 100 per cent conversion

DISCUSSION

Although there is a marked increase in the storage of sugar as glycogen under the influence of insulin, less than a quarter of the extra sugar which is retained is stored in this manner. The fate of the remainder is uncertain. One possibility is that it may have been oxidized, but it would be almost inconceivable that the quantity concerned (1.8 gm. per sq. dm. of body surface) could be thus accounted for, as this would necessitate an increase of 48 per cent (corrected for the reduction in protein catabolism) in the total metabolic rate. Diabetic rats such as were used in this study have no basal insulin requirement and maintain their weight on the high carbohydrate diet. Without insulin they do not add to their fat deposits nor do they deplete them. Insulin causes them to eat somewhat less and makes them retain more sugar and gain weight, findings which would not point toward a significant increase in glucose oxidation. Furthermore, insulin has not been found (5) to increase the metabolic rate of normal rats. To examine this point under our conditions oxygen consumption measurements were made over a period of hours upon three of the diabetic rats with and without insulin. The average for the controls was 1.3 cc. and for the insulin-treated animals 1.2 cc. of oxygen per sq. dm. of body surface per minute.

Another possible disposition of glucose under the influence of insulin is that it may enter into protein synthesis (12, 13). In the present experiments nitrogen retention by the organism was promoted by insulin. However, if all of the carbon in protein formed by the extra nitrogen retained owing to insulin came from the glucose, it would account for only 8 per cent of the stored sugar.

Macleod (1) suggested that insulin might cause glucose to be stored in the form of some substance related to hexose phosphate which is not precipitated as glycogen but which at the same time has lost its reducing properties. Recent discoveries concerning the mechanism of glycogenesis and glycolysis (14) enhance this possibility and it is desirable that quantitative studies of the extent of glucose storage as glucose-1-phosphate, etc., be made. It should be pointed out that the conversion of all of the stored glucose, which cannot be accounted for in this study, to glucose-1-phosphate would require a very large amount of phosphoric acid.

The storage of carbohydrate as glucose and glycogen is very limited and any appreciable amount of carbohydrate which cannot be promptly catabolized under normal conditions is stored as fat (15). It would seem most likely that the storage of glucose due to insulin is largely in the form of fat. Macleod (16) first suggested that fat-like substances might be produced by the action of insulin on glucose. DeNayer (17), working in Bouckaert's laboratory, found that when just the amount of glucose needed to keep the blood sugar level normal was given to insulin-treated rabbits

there was no increase in muscle glycogen, although large amounts of glucose were disposed of in the body. Bouckaert² suggested that the latter was transformed into fat and that one of the actions of insulin is to increase the conversion of glucose to fat. The view (4) that fat is the great energy reservoir of the body and one of the most important functions of insulin is to facilitate the formation of fat from carbohydrate and thus to store the latter in its most efficient form is rapidly gaining support. The most likely possibility is that the sugar was stored by the action of insulin in our experiments largely in the form of fat. This question should be pursued further.

SUMMARY

Insulin administration causes diabetic rats to store large quantities of sugar. There are large increases in the glycogen content of the liver and muscles but only 15 and 9 per cent respectively of the stored sugar is accounted for by the extra glycogen in these tissues.

There is no evidence that any appreciable part of the sugar which is not laid down as glycogen is oxidized. Only a negligible amount could be accounted for by possible protein synthesis. Transient storage as carbohydrate metabolites must be considered. Most ingested carbohydrate is normally stored as fat and it is considered the most likely possibility that this is the fate of the bulk of the sugar stored under the influence of insulin.

BIBLIOGRAPHY

1. Macleod, J. J. R., *Medicine*, **3**, 223 (1924).
2. Cori, C. F., *Physiol. Rev.*, **11**, 143 (1931).
3. Soskin, S., *Physiol. Rev.*, **21**, 140 (1941).
4. Drury, D. R., *Am. J. Physiol.*, **131**, 536 (1940-41).
5. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **70**, 557 (1926).
6. Best, C. H., Hoet, J. P., and Marks, H. P., *Proc. Roy. Soc. London, Series B*, **100**, 32 (1926).
7. Shapiro, R., and Pincus, G., *Proc. Soc. Exp. Biol. and Med.*, **34**, 416 (1936).
8. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 455 (1933).
9. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
10. Shaffer, P. A., and Hartmann, A. F., *J. Biol. Chem.*, **45**, 365 (1920-21).
11. Drury, D. R., Bergman, H. C., and Greeley, P. O., *Am. J. Physiol.*, **117**, 323 (1936).
12. Janney, N. W., and Shapiro, I., *Arch. Int. Med.*, **38**, 96 (1926).
13. MacKay, E. M., Barnes, R. H., and Bergman, H. C., *Am. J. Physiol.*, **126**, 155 (1939).
14. Cori, C. F., in Lewis, H. B., *Biological symposia*, Lancaster, **5**, 131 (1941).
15. MacKay, E. M., and Drury, D. R., *Am. J. Physiol.*, **132**, 661 (1941).
16. Macleod, J. J. R., *Proceedings of the Institute of Medicine of Chicago*, Feb. (1923).
17. DeNayer, P., *Arch. internat. pharmacod. et therap.*, **42**, 461 (1932).

² Bouckaert, J., personal communication.

THE ERGOT ALKALOIDS

XIX. THE TRANSFORMATION OF *dl*-LYSERGIC ACID AND *d*-LYSERGIC ACID TO 6,8-DIMETHYLERGOLINES

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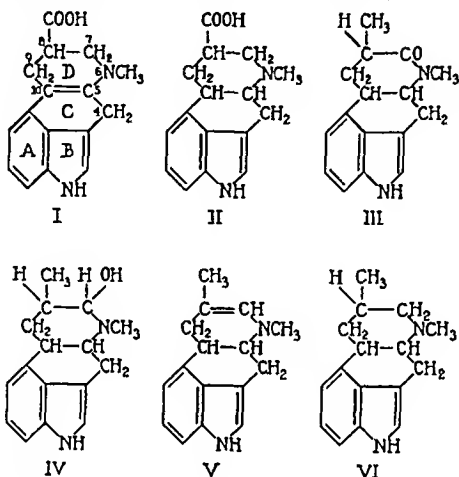
In a former paper (1), the production of a base from *d*-lysergic acid and its comparison with synthetic 6,8-dimethylergoline have been described. Although agreement in general properties was observed, a 15–20° lower melting point was obtained with the base prepared from *d*-lysergic acid. The yield in the final step of the series of reactions beginning with α -dihydrolysergic acid was very low and the question of homogeneity remained uncertain. It was, therefore, important to continue the study and to attempt the transformation in a manner which would increase the yield of final product. This has since been accomplished, but complications of a stereochemical character have naturally been encountered. 6,8-Dimethylergoline (VI) possesses three centers of asymmetry, and the synthetic substance which we have described, if homogeneous, could consist of any one of four possible racemic pairs. Otherwise, it would be a mixture of at least two or more of such possible racemic pairs. On the other hand, the substance obtained from optically active α -dihydrolysergic acid (II) could be also of optically active character and could, therefore, have different physical properties. This has been found to be the case. Therefore, it was advisable to turn to racemic or *dl*-lysergic acid as the starting point for the same transformations. This procedure has proved successful.

dl-Lysergic acid was prepared according to the method of Stoll and Hofmann (2) by cleavage of ergotinine with hydrazine, followed by hydrolysis of the resulting hydrazide (of *dl*-isolysergic acid) by alkali. The resulting racemic lysergic acid was reduced with sodium and butyl alcohol¹ to a *dl*-dihydrolysergic acid as in the case of *d*-lysergic acid (3).

On sublimation under reduced pressure, this substance was converted into the *unsaturated dl*-lactam, $C_{15}H_{16}ON_2$, which on hydrogenation gave in turn the *saturated dl*-lactam, $C_{15}H_{18}ON_2$, (4) (III). On recrystallization of the latter, there was evidence that at least partial separation into racemic modifications had occurred. The last step of hydrogenation reestablishes asymmetry at carbon atom 8. One of these fractions had a melting point as high as 332–336°, and material was obtained from the mother liquors which melted

¹ The amyl alcohol as given in this older work (3) has since been replaced by normal butyl alcohol.

at 310–315°. No depression in melting point was apparent on mixing the two fractions, and the question of the homogeneity of the lower melting substance was not further investigated. Because of the scarcity of material these fractions were recombined for the final step of reduction with sodium and butyl alcohol. It was at this point that our recent experience with optically active α -dihydrolysergic acid, as described below, became the guide for a better understanding of the course of this reaction.



6,8-Dimethylergoline, $C_{16}H_{20}N_2$, (VI) had been found to be a very uncertain and minor product of the reaction. It now appears that the corresponding hydroxy derivative, *viz.* 6,8-dimethyl-7-hydroxyergoline, $C_{16}H_{20}ON_2$, (IV) must be the main reduction product of the reaction and on sublimation for purification loses water to produce the unsaturated *dehydro-dl*-6,8-dimethylergoline, $C_{16}H_{18}N_2$, represented in Formula V. There is the possibility that this unsaturated substance could be formed at least in part under the conditions of the reaction with sodium and butyl alcohol before sublimation. Its further partial reduction at the double bond could then have produced the small yield of dimethylergoline already reported as a direct product from the hydrogenated lactam prepared from α -dihydrolysergic acid.

The exact position of the double bond of *dehydro-dl*-6,8-dimethylergoline could be any one of the positions between carbon atoms 7,8, 8,9, or possibly 9,10. This is a point which remains to be established by later work. When this unsaturated substance was hydrogenated, a product was obtained in good yield which gave the correct analytical results for a dimethylergoline. The color reactions of the latter were indistinguishable from those shown by synthetic 6,8-dimethylergoline. The melting points

(micro) also proved to be indistinguishable ($227-229^{\circ}$), and no depression was shown by the mixture of the two substances. The crystalline form of the substances obtained from both sources showed a close resemblance, as will be noted in Fig. 1. These results, therefore, confirm the structure of lysergic acid shown in Formula I, which was derived from the study of its degradation (5).

When the optically active α -dihydrolysergic acid was employed for the above series of reactions, the melting point of the substance finally obtained for comparison with synthetic dimethylergoline was found to be higher

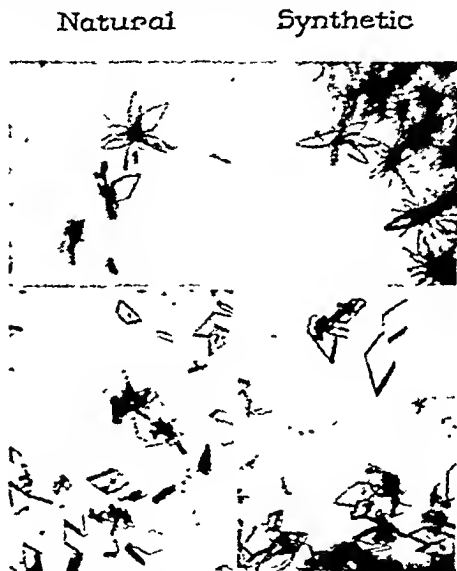


Fig. 1. Comparison of the dimethylergolines

than that originally reported (1). As previously recorded, α -dihydrolysergic acid was pyrolyzed to the unsaturated lactam, which was in turn hydrogenated to the saturated lactam. The latter on recrystallization has now been separated into a higher melting ($332-336^{\circ}$) substance, and a more soluble, apparently isomeric and lower melting material ($300-305^{\circ}$) of doubtful homogeneity. The higher melting substance was reduced with sodium in butyl alcohol. Although the product isolated from the reaction mixture could be directly crystallized from benzene, it proved to be unstable. It was, therefore, sublimed under low pressure. The sublimate yielded a well characterized crystalline substance which melted at $155-157^{\circ}$. Analysis showed this substance to be apparently one of the possible

stereoisomers of the unsaturated dehydro-6,8-dimethylergoline, $C_{16}H_{18}N_2$. This in turn on hydrogenation gave an optically active dimethylergoline, $C_{16}H_{20}N_2$, which melted at 246–248°. The lower melting (m.p. 205–212°) base previously reported, and shown to be optically active as the hydrochloride, was therefore apparently still a mixture of stereoisomers.

When the lower melting (300–308°) and apparently isomeric saturated lactam mentioned above was reduced with sodium and butyl alcohol, a somewhat different behavior was noted from that shown by the higher melting (336°) saturated lactam. A mixture of products was obtained after sublimation, analysis of which suggested a mixture of a dimethylergoline and dehydrodimethylergoline. From this, on recrystallization, a dimethylergoline could be isolated but in an amount too small for extended recrystallization. It appears that this could have been the source of the dimethylergoline originally reported (1). Stereochemical relationships on carbon atoms 7 and 8 could perhaps account for the differences in behavior which were noted with the saturated lactams during reduction and sublimation.

In this connection, the occasion is taken to record an isomeric *unsaturated lactam*, $C_{16}H_{16}ON_2$, which has been prepared from γ -dihydrolysergic acid (6).

EXPERIMENTAL

dl-Lysergic Acid—Ergotinine was converted into *di*-isolysergic hydrazide and the latter was hydrolyzed to *dl*-lysergic acid, as described by Stoll and Hofmann (2). From 20 gm. of ergotinine, 3.5 gm. of the colorless hydrazide were obtained, which melted at 240–241° with decomposition. The melting point depended to a considerable extent on the rate of heating. Stoll and Hofmann reported 240°.

$C_{16}H_{18}ON_4$. Calculated, C 68.04, H 6.43; found, C 68.04, H 6.39

3.2 gm. of the hydrazide were refluxed with 15 cc. of alcohol and 15 cc. of 25 per cent KOH for 1 hour. After being cooled to 0°, the solution was brought almost to the neutral point with HCl and then acidified with excess acetic acid. The suspension of finely divided precipitate was carefully brought to a boil and then cooled and the precipitate was collected with water. The yield of practically pure, colorless *dl*-lysergic acid was 2.67 gm., or 88 per cent. The macro melting point was 251° with decomposition. Stoll and Hofmann reported 240–250° with decomposition. The material showed no optical activity.

$C_{16}H_{16}O_2N_2$. Calculated, C 71.60, H 6.02; found, C 71.49, H 6.02

dl-Dihydrolysergic Acid—The reduction of *dl*-lysergic acid was carried out essentially as previously described for lysergic acid itself (3), but with butyl alcohol as solvent. However, a simpler procedure was used for

isolating the reduction product. The aqueous alkaline solution, after removal of the butyl alcohol, was cooled to 0° and carefully made acid to Congo red with HCl, and then brought back to the alkaline side with ammonia. The solution was boiled for a few minutes and allowed to stand in the cold. The precipitate was collected with water and washed thoroughly with alcohol. From 2.67 gm. of *dl*-lysergie acid, 1.67 gm. of *dl*-dihydrolysergie acid were obtained. The substance was further purified by treating an ammoniacal solution with norit followed by concentration to the point of crystallization. It formed diamond-shaped platelets from water which decomposed without melting at $290\text{--}300^{\circ}$.

$C_{16}H_{15}O_2N_2$. Calculated, C 71.06, H 6.72; found, C 71.12, H 6.80

Unsaturated Lactam from dl-Dihydrolysergie Acid—Sublimation of *dl*-dihydrolysergie acid at 350° under a pressure of 25 mm. was carried out as previously described for *d*-lysergie acid (4). Four runs with a total of 2.23 gm. yielded 0.69 gm. of unsaturated lactam. The crude product was decolorized with norit in methyl alcohol. It formed needles which melted at $313\text{--}316^{\circ}$.

$C_{16}H_{14}ON_2$. Calculated, C 76.15, H 6.41; found, C 75.83, H 6.79

Saturated Lactam from dl-Dihydrolysergie Acid—160 mg. of the unsaturated lactam were hydrogenated with 50 mg. of platinum oxide catalyst in 13 cc. of glacial acetic acid. In 35 minutes the theoretical volume of hydrogen for 1 mole had been absorbed and the hydrogenation was interrupted. After removal of the catalyst, the solution was concentrated until crystallization began. The filtrate was evaporated to dryness and a second crop of crystals was obtained from methyl alcohol. In this way about 140 mg. of flat needles or tightly packed leaflets were obtained which gave the proper analytical figures.

$C_{16}H_{17}ON_2$. Calculated, C 75.54, H 7.14; found, C 75.54, H 7.12

Fractional crystallization gave some material with a melting point as high as $332\text{--}336^{\circ}$ (found, C 75.50, H 7.25) and some as low as $310\text{--}315^{\circ}$. A mixed melting point of the two fractions showed no depression, and it is likely that they are different racemic modifications. For the next step these fractions were, however, recombined.

Dehydro-dl-6,8-dimethylergoline—140 mg. of the saturated lactam were reduced with sodium and butyl alcohol as described below for the optically active substance. It was possible to obtain crystalline material directly from the reaction product before sublimation, but the melting point was not sharp ($175\text{--}185^{\circ}$), and attempted recrystallization did not help much. Accordingly, the entire reaction product was sublimed under 0.2 mm.

pressure. A total of 93 mg. of sublimate was collected up to a bath temperature of 200°. Upon recrystallization from methyl alcohol, 40 mg. of well formed, stout needles were obtained which melted at 182-186°.

$C_{16}H_{18}N_2$. Calculated, C 80.64, H 7.62; found, C 80.10, H 7.38

The analysis approached that required for the unsaturated 6,8-dimethylergoline. The carbon figures were somewhat low. However, the crystalline material was directly reduced to the succeeding saturated base without further attempts at purification.

dl-6,8-Dimethylergoline—The above base was hydrogenated with 20 mg. of platinum oxide catalyst in 2 cc. of glacial acetic acid. Absorption proceeded very rapidly and in 5 minutes was already well past the 1 mole stage. It was interrupted and the catalyst was filtered off. The filtrate was evaporated to dryness and the residue treated with a little water followed by sodium hydroxide. The precipitated base was extracted with ether. After drying over potassium carbonate, the solution was concentrated until crystallization began, and then chilled. 15 mg. of the product were collected with ether and melted at 220-224°.

Recrystallization of this material from methyl alcohol gave 13 mg. of large, diamond-shaped or boat-shaped plates which melted at 227-229°. Further recrystallization from the same solvent did not appreciably change the melting point.

$C_{16}H_{20}N_2$. Calculated, C 79.94, H 8.39; found, C 80.04, H 8.31

When the melting point was taken very slowly, two types of crystals could be detected under the microscope near the melting point and these appeared to have a different melting point. When the melted material was allowed to cool, the first crystals to be seen appeared to be a mass of needles or rods which began to melt again at about 220°. Broad plates then appeared in the melt and these plates did not entirely disappear until a temperature of 231° was reached. The synthetic material (1) was found to behave in similar fashion. A mixed melting point with the synthetic material did not show a depression, and the crystalline form gave every indication of identity. The characteristic Keller color test was indistinguishable for the two substances.

Dehydro-6,8-dimethylergoline Derived from α -Dihydrolysergic Acid—860 mg. of α -dihydrolysergic acid were pyrolyzed in two separate runs to the unsaturated lactam as previously reported (4). This yielded 200 mg. of material with the highest melting point, and somewhat more material with a lower melting point. The latter was set aside. The former was hydrogenated to the saturated lactam (4), except that in this case ethyl alcohol was used as the solvent instead of acetic acid. The absorption of hydrogen was considerably slower than in acetic acid and it was necessary to warm

the hydrogenation flask to complete the reaction. Crystalline material separated out at room temperature. Even then hydrogenation appeared to stop completely at a point just short of the absorption of a full mole of hydrogen. The catalyst was filtered off from the warm solution and the material in the filtrate was subjected to fractional crystallization. This resulted in a total yield of 71 mg. of saturated lactam as diamond-shaped leaves which melted at temperatures varying from 332–336°.

$C_{16}H_{15}ON_2$. Calculated, C 75.54, H 7.14; found, C 75.57, H 7.00

From the mother liquors, 70 mg. of additional material which did not melt sharply at 300–308° were obtained. This material was slightly more soluble in the various solvents than the first reaction product. It gave, however, excellent analytical results, which indicated stereoisomeric material. Found C 75.45, H 7.22.

The high melting fraction was dissolved in 20 cc. of dry butyl alcohol and heated to boiling. 1 gm. of sodium was added, and the molten sodium was well dispersed by vigorous shaking. After disappearance of all sodium, water was added and the butyl alcohol was removed under reduced pressure. The insoluble base which remained was extracted with ether and the ether solution was dried over K_2CO_3 . Crystalline material, which melted at approximately 140°, could be obtained at this point but the solution became progressively colored, and the crystals were not sharply defined. Accordingly, the above ether solution was evaporated to dryness in a sublimation apparatus, and the residue was sublimed. A first fraction of 35 mg. was collected rapidly at 160–170° under 0.2 mm. pressure. A second fraction of 15 mg. sublimed up to 220°. The first fraction had an indefinite melting point of approximately 150°, and on analysis was found to be somewhat low in carbon for the unsaturated dehydro-6,8-dimethylergoline.

$C_{16}H_{14}N_2$. Calculated, C 80.64, H 7.62; found, C 80.06, H 7.75

This fraction crystallized nicely from benzene and yielded 17 mg. of well formed, heavy, elongated crystals which melted at 155–157°, and now gave satisfactory analytical figures for the dehydro-6,8-dimethylergoline. Found, C 80.57, H 7.43.

6,8-Dimethylergoline from α -Dihydrolysergic Acid—The above crystalline material was recombined with its mother liquor and with the second fraction which sublimed up to 220°. This gave a total of 40 mg. of solid crude material. The mixture was hydrogenated in 2 cc. of glacial acetic acid with 20 mg. of platinum oxide catalyst. In 12 minutes, the theoretical volume of hydrogen for 1 mole had been absorbed and the hydrogenation was interrupted. The filtrate, after evaporation, gave a residue which was dissolved in a little water. The base was precipitated with alkali

and extracted with ether. On concentration of the dried extract to a small volume, 7 mg. of crystalline base separated which melted at 228–236° with a change in crystalline form apparent at about 170°. The mother liquor on concentration gave a residue which yielded from acetone 4.5 mg. of additional crystalline material which melted at the same point as did the first fraction. It was therefore combined with the latter for recrystallization from acetone. 6 mg. of well formed heavy polyhedrons with square ends were obtained. A few rods were also present. The material appeared to change its crystalline form at approximately 170° to needles which melted at 246–248°.

$$[\alpha]_D^{25} = -49^\circ \text{ (} c = 0.286 \text{ in chloroform)}$$

$C_{16}H_{20}N_2$. Calculated, C 79.94, H 8.39; found, C 79.87, H 8.26

Reduction of Isomeric Saturated Lactam from α -Dihydrolysergic Acid—When the lower melting dihydro lactam (70 mg., m.p. 300–308°) was treated exactly as in the case of the higher melting material, it behaved somewhat differently. Crystalline material could not be obtained before sublimation. 25 mg. sublimed up to a temperature of 200° under 0.2 mm. pressure, and 13 mg. more up to a temperature of 250°. The sublimate was dissolved in benzene, treated with a little bone-black to remove color, and the filtrate was concentrated to a small volume. 9 mg. of crystalline material were collected which melted, but not sharply, at 200–204°. It showed the characteristic change of crystalline form before melting already noted with the above 6,8-dimethylergoline. When recrystallized from acetone, it yielded 2.5 mg. of the same type of crystals. However, the melting point was lower, 234–238°.

$C_{16}H_{20}N_2$. Calculated, C 79.94, H 8.39; found, C 80.02, H 8.27

Unsaturated Lactam from γ -Dihydrolysergic Acid— γ -Dihydrolysergic acid, prepared from ergotinine (6), was pyrolyzed by sublimation at 25 mm. and 350°, as previously described. The product, after recrystallization from a small volume of methyl alcohol formed long needles which melted at 239–240°.

$$[\alpha]_D^{25} = -197^\circ \text{ (} c = 0.517 \text{ in pyridine)}$$

$C_{16}H_{18}ON_2$. Calculated, C 76.15, H 6.40; found, C 75.70, H 6.53

BIBLIOGRAPHY

1. Jacobs, W. A., and Gould, R. G., Jr., *J. Biol. Chem.*, **130**, 399 (1939).
2. Stoll, A., and Hofmann, A., *Z. physiol. Chem.*, **250**, 7 (1937).
3. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **106**, 393 (1934).
4. Jacobs, W. A., and Craig, L. C., *J. Am. Chem. Soc.*, **60**, 1701 (1938).
5. Craig, L. C., Shedlovsky, T., Gould, R. G., Jr., and Jacobs, W. A., *J. Biol. Chem.*, **125**, 289 (1938).
6. Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **115**, 227 (1936).

SYNTHESIS OF A 3,4-DIAMINOTETRAHYDROTHIOPHENE AND A COMPARISON OF ITS STABILITY WITH THE DIAMINO-CARBOXYLIC ACID DERIVED FROM BIOTIN*

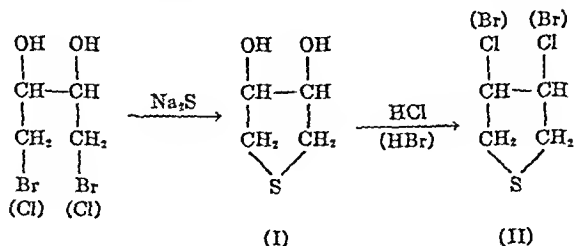
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Early in the work on the structure of biotin in this laboratory it became evident that one of the most likely possibilities (1) for the basic ring system of this vitamin was the cyclic urea derived from a 3,4-diaminotetrahydrothiophene. In the course of the degradation work it had been reported that the urea ring could be cleaved with the formation of a diamino compound (2, 3). It therefore became of interest to study the stability and certain other properties of 3,4-diaminotetrahydrothiophene. As the work developed, we became particularly interested in the comparison of the absorption spectrum of the dibenzoquinoxaline derivative of the model with that of the corresponding biotin derivative. This latter aspect will be dealt with in a subsequent presentation.

The first approach to the synthesis of 3,4-diaminotetrahydrothiophene was through the corresponding dihalo compounds which were prepared as shown in the accompanying equations (Formulas I and II). The structure

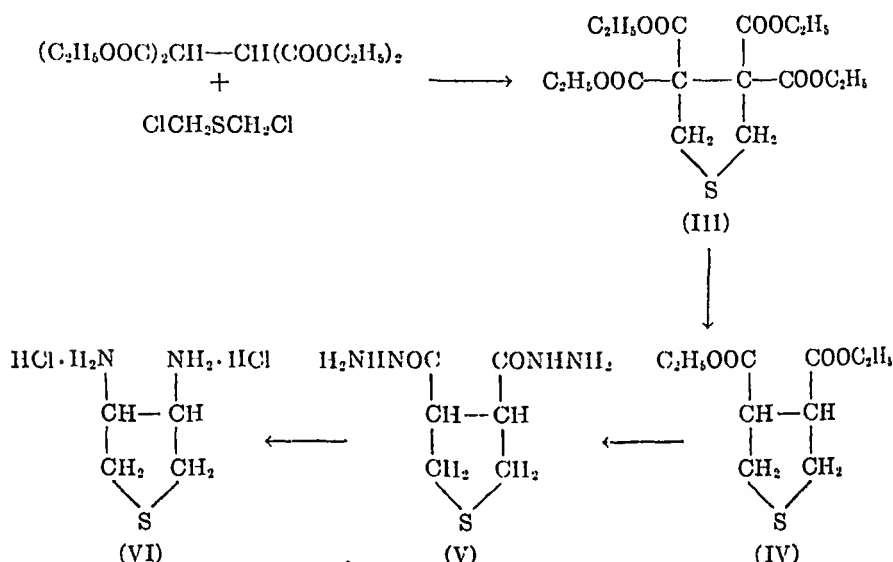


expressed by Formula II was indicated by the formation of a typical thiophene color reaction with isatin and sulfuric acid when the material was heated with alcoholic KOH, and in the case of the dibromo compound by oxidation to the sulfone, which was found to be identical with an authentic sample prepared by addition of bromine to 2,5-dihydrothiophene sulfone

* The authors wish to express their appreciation to the S. M. A. Corporation and to Merck and Company, Inc., for supplies of biotin.

(4,5). However, all efforts to replace the halogen atoms of Compound II by the use of ammonia, potassium phthalimide, and various other reagents were unsuccessful. Similar attempts to replace the bromine atoms of the sulfone were also unsuccessful.

The desired diamine was obtained by the accompanying reactions (Formulas III to VI).



Ethyl 3,3,4,4-tetrahydrothiophenetetracarboxylate (III) was prepared according to the method of Mann and Pope (6). The tetraester was saponified, decarboxylated, and reesterified to the diester (IV), which was converted to the crystalline dihydrazide (V). This compound was rearranged by the Curtius method and the diurethane hydrolyzed to the diamine salt (VI). This material was characterized through its crystalline dipicrate, dibenzoyl, diacetyl, and dibenzoquinoxaline derivatives; the latter derivative demonstrates that the compound is a 1,2-diamine. On being heated with phosphoric acid the amine salt evolved vapors which gave a typical thiophene color with isatin and sulfuric acid.

Whereas biotin can be resynthesized in almost quantitative yield (7) from the diamino compound by treatment with phosgene and NaOH, the diamine (VI) when treated similarly did not yield the corresponding cyclic urea derivative. The action of diethyl carbonate (8) on the free diamine also failed to yield a carbonyl derivative. Whether or not failure to form this cyclic derivative is due to the steric arrangement of the amino groups is under investigation.

Since the sulfur of biotin is relatively stable to the action of hydriodic acid (9), a qualitative comparison of the stability of the simple tetrahydrothiophene compound with that of the diaminocarboxylic acid from biotin was made. The hydrogen sulfide and ammonia produced by the action of hydriodic acid or of alkali at various temperatures were used as criteria. When heated for 2 hours at 210° with fuming hydriodic acid, both the 3,4-diaminotetrahydrothiophene and biotin were unchanged. However, under the still more vigorous conditions of treatment for 5 hours at 250° with the fuming hydriodic acid the 3,4-diaminotetrahydrothiophene gave up 5 to 10 per cent of its sulfur as hydrogen sulfide and 10 to 15 per cent of its nitrogen as volatile base. Under these latter conditions the diaminocarboxylic acid derived from biotin liberated 50 per cent of its sulfur and 80 to 90 per cent of its nitrogen.

Of considerable interest is the fact that the dihydroxy compound (I) yielded large quantities of hydrogen sulfide with hydriodic acid at 210°. It may be recalled that Kōgl and de Man (9) had observed liberation of hydrogen sulfide upon similar treatment of an oil which they believed to be the diol corresponding to the diaminocarboxylic acid from biotin.

Qualitatively no inorganic sulfide formation has been observed in the treatment of biotin with alkaline reagents (2, 3). Treatment of 3,4-diaminotetrahydrothiophene or its diacetyl derivative in the same manner also failed to produce sulfide, and the ammonia formation was relatively low.

Thus the stability of the 3,4-diaminotetrahydrothiophene is not incompatible with the presence of this moiety in the diaminocarboxylic acid derivable from biotin. The greater instability of the diaminocarboxylic acid under the very drastic hydriodic acid treatment may be due to the influence of the side chain, but the possible influence of steric configuration must be borne in mind.

EXPERIMENTAL

3,4-Dichlorotetrahydrothiophene—9.3 gm. of *dl*-1,4-dibromo-2,3-dihydroxybutane (10) were dissolved in 50 cc. of water at 60–70° and 18 gm. of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ in 5 cc. of water were added in portions with stirring, the reaction mixture being kept at 50–60°. The mixture was then heated for 2 hours on a steam bath. After acidification to Congo red with 20 per cent HCl, the solvent was removed *in vacuo* and the almost dry residue was extracted six times with absolute ethanol. Evaporation of the alcohol *in vacuo* left a viscous pink oil and an inorganic salt; a large portion of the latter was removed by solution in a small volume of absolute ethanol, filtration, and reevaporation. The oily residue was taken up in 17.5 cc. of concentrated HCl and was heated in a sealed tube for 5 hours at 150°.

Steam distillation of the black, tarry product yielded 1.9 gm., representing 32 per cent of the theoretical yield, of a white solid, melting at 57–59°.¹ When purified by re crystallization from petroleum ether, the material was obtained as clusters of white needles, melting at 60–61°. When a small sample was boiled with 50 per cent KOH in alcohol and the vapors led through isatin-H₂SO₄, a green coloration similar to that from thiophene was observed.

C₄H₆Cl₂ (157.1). Calculated, S 20.41; found, S 20.09

3,4-Dibromotetrahydrothiophene—10 gm. of *dl*-1,4-dibromo-2,3-dihydroxybutane (10) were treated with Na₂S as in the preparation of 3,4-dichlorotetrahydrothiophene. The residue from the alcohol extractions was dissolved in 20 cc. of aqueous HBr (saturated at 0°) and the solution was heated on a steam bath under a reflux condenser for 5 hours. The badly darkened material was then distilled with steam. The oil in the distillate was separated by chloroform extraction. The solution was dried and concentrated and the residual oil was distilled at 3 mm.; 2.5 gm., representing 25 per cent of the theoretical yield, of an almost colorless oil were obtained at 83–89°.

A small sample of the sulfide was oxidized to the sulfone. It was shaken for 30 minutes with saturated aqueous permanganate in the presence of acetic and sulfuric acids (11). After re crystallization the sulfone melted at 137–142°. A mixture with an authentic specimen (4, 5) of 3,4-dibromotetrahydrothiophene sulfone, which melted at 137–142°, showed no depression of the melting point.

1,4-Dichloro-2,3-dihydroxybutane—20 gm. of 1,4-dichloro-2-butene (12) were oxidized to the diol with dilute permanganate by the procedure used by Thiele (10) on 1,4-dibromo-2-butene. The yield was about 35 per cent of the theoretical amount. The product can be distilled at 113–118° at a pressure of 4 mm. and after several recrystallizations from benzene-hexane it melted at 62–63°. This compound is apparently a diastereoisomer of the erythritol dichlorohydrin, melting at 126°, which was previously reported (13, 14).

C₄H₈O₂Cl₂ (159.0). Calculated, C 30.21, H 5.04; found, C 29.88, H 5.21

3,4-Dihydroxytetrahydrothiophene—4.9 gm. of the 1,4-dichloro-2,3-dihydroxybutane in 35 cc. of water were treated with Na₂S in the same manner as the corresponding bromo compound. The material which was extracted with absolute alcohol was relatively free from inorganic salts. An obviously impure compound crystallized and was extracted from extraneous material with chloroform. After the chloroform was evaporated, the

¹ Melting points are uncorrected.

residue was dried over P_2O_5 and the solid sublimed in a molecular still in small batches, at 3 to 4 mm. and a bath temperature of 95° . In this way 1.9 gm. of material were obtained, representing 51 per cent of the theoretical yield. Several resublimations of a small sample yielded clusters of fine prisms, which melted at $54-58^\circ$.

$C_8H_6O_2S$ (120.2). Calculated, S 26.63; found, S 25.73

Ethyl Tetrahydrothiophene-3,3,4,4-tetracarboxylate—According to a modification of the method of Mann and Pope (6), 7.2 gm. of clean sodium were dissolved in 700 cc. of absolute alcohol and 50 gm. of ethyl ethane-1,1,2,2-tetracarboxylate were added. The solution was refluxed for 1 hour, cooled to $40-50^\circ$, and 21 gm. of bis(chloromethyl) sulfide were added; the mixture was then heated under a reflux for 3 hours. After removal of the NaCl, the filtrate and washings were concentrated *in vacuo*. The residue was washed with water, dried, and was distilled. After a small fore run, which was discarded, 22 gm. of oil, boiling at $192-200^\circ$ at a pressure of 8 mm., were obtained. This material deposited a small amount of ethyl ethanetetra-carboxylate when the fraction was cooled. A further fraction boiling at $200-208^\circ$ at a pressure of 8 mm. weighed 16 gm. and deposited no solid. The English workers report a boiling point of $220-223^\circ$ at a pressure of 15 mm. but do not indicate the yield obtained.

Tetrahydrothiophene-3,4-dicarboxylic Acid Dihydrazide—2.8 gm. of the tetraester were heated in a bath at 80° for about 30 hours with 37 cc. of 1 N NaOH. Titration of the excess alkali indicated that about 95 per cent of the theoretical quantity of alkali had been consumed. The alkaline solution was extracted with ether, made quite strongly acid, and again extracted with ether. After evaporation of the ether the residue was heated for about 9 hours in a bath at $140-160^\circ$ to eliminate CO_2 . The viscous residue was then esterified at room temperature with ethanol and HCl. After evaporation of the alcohol the oil was dissolved in ether, washed with water, dried, and evaporated. The residue was 2.8 gm. of a pleasant smelling oil. To it were added 1.8 gm. of hydrazine hydrate, and the mixture was heated for 3 hours in a boiling water bath. The cooled mixture of oil and solid was drained on a filter and was washed with alcohol. The residue was dissolved in water and was extracted with benzene. The aqueous solution was treated with decolorizing carbon and the filtrate concentrated to about 2 cc. 2 volumes of absolute alcohol were added and the product which precipitated as fine needles was collected and was recrystallized from water-alcohol. It weighed 265 mg., which represents 17 per cent of theoretical yield from the tetraester. The compound melted at $226-227^\circ$, softening at a somewhat lower temperature.

$C_8H_{12}O_2N_2S$ (204.2). Calculated, S 15.70; found, S 15.55

The dihydrazide was also obtained in 7 per cent yield by partial hydrolysis of the tetraester at room temperature with 2 moles of 0.1 N NaOH. The crude diester-diacid was decarboxylated and the diester treated with hydrazine as above.

3,4-Bis(carboethoxyamino)tetrahydrothiophene—2.2 gm. of the dihydrazide were dissolved in 29 cc. of 1 N HCl and the solution cooled in ice and covered with 60 cc. of ether (15). During about 10 minutes 1.5 gm. of NaNO_2 were added in portions with mechanical stirring. The ether was separated and the cold aqueous phase extracted four times with 80 cc. portions of ether. After the ether was dried over Na_2SO_4 at 0° , it was filtered and the salt was washed with cold ether. The ether filtrate, which amounted to 900 cc., was diluted with 1 liter of ice-cold absolute alcohol. The solution was placed in a cold water bath and the ether distilled during about 5 hours by gentle heating. The alcohol was then evaporated *in vacuo*. The residue was dissolved in methanol, was decolorized with charcoal, and the filtrate was evaporated. The crude diurethane weighed 2.5 gm., which is 88 per cent of the theoretical yield from the dihydrazide. The melting point was $159\text{--}163^\circ$. When purified by several recrystallizations from alcohol-water, it formed beautiful white blades, melting at $176\text{--}178^\circ$.

$\text{C}_{16}\text{H}_{18}\text{O}_4\text{N}_2\text{S}$ (262.3). Calculated, S 12.22; found, S 12.30

3,4-Diaminotetrahydrothiophene Dihydrochloride—700 mg. of crude diurethane were heated for 3 hours with 12.5 cc. of concentrated HCl in a sealed tube at $100\text{--}105^\circ$ (15). The dark solution was decolorized with carbon, and the filtrate was concentrated to 20 cc. *in vacuo*. The white solid that separated when the solution was cooled was collected and washed with alcohol and ether. This material gave no qualitative test for ammonium salts. It amounted to 288 mg., which is 58 per cent of the theoretical yield from the urethane. It was dissolved in a small volume of water and was precipitated by the addition of 2 volumes of dioxane. The compound begins to decompose at about 250° and gave the following analysis.

$\text{C}_4\text{H}_{12}\text{N}_2\text{Cl}_2\text{S}$ (191.1). Calculated, S 16.77; found, S 16.60

A small sample of the dihydrochloride was heated with H_3PO_4 and the vapors led through isatin- H_2SO_4 . A beautiful blue-green color, very similar to that produced when thiophene is treated likewise, was produced.

The diamine formed a dipierate which was very insoluble in water or alcohol. The compound begins to decompose at about 250° .

$\text{C}_{16}\text{H}_{16}\text{N}_4\text{O}_4\text{S}$ (576.4). Calculated, S 5.56; found, S 5.18

Treatment with benzoyl chloride and NaOH gave a dibenzoyl derivative which crystallized from acetic acid-water as fine white prisms, which melted at 295–300°, but softened somewhat lower.

$C_{19}H_{15}O_2N_2S$ (326.4) Calculated, S 9.82; found, S 9.44

Treatment of the diamine with acetic anhydride and NaOH yielded a water-soluble diacetyl derivative. It crystallized from methanol-ether as white needles, which sublimed on a micro melting point stage at 260–265°.

$C_8H_{11}O_2N_2S$ (202.3) Calculated, N 13.86; found, N 13.63

The dibenzoquinovoline derivative of this diamine is described in the following paper.

The free diamine was obtained in crude form by extraction of a cooled solution of the dihydrochloride in 50 per cent NaOH with chloroform. Evaporation of the chloroform left impure crystals, which melted at about 40°.

The authors wish to express their appreciation to Dr. Julian R. Rachel of this laboratory for carrying out the microanalyses.

SUMMARY

A 3,4-diaminotetrahydrothiophene has been synthesized, and its stability toward hydriodic acid and toward alkali has been compared with that of the diaminocarboxylic acid derived from biotin.

BIBLIOGRAPHY

1. du Vigneaud, V., Hofmann, K., and Melville, D. B., *J. Am. Chem. Soc.*, **64**, 188 (1942).
2. Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **141**, 207 (1941).
3. Kögl, F., and Pons, L., *Z. physiol. Chem.*, **269**, 61 (1941).
4. Staudinger, H., and Ritzenthaler, B., *Ber. chem. Ges.*, **68**, 455 (1935).
5. van Zuydewijn, E. de R., *Rec. trav. chim. Pays-Bas*, **57**, 445 (1938), *Chem. Abst.*, **32**, 6617 (1938).
6. Mann, F. G., and Pope, W. J., *J. Chem. Soc.*, **123**, 1172 (1923).
7. Melville, D. B., Hofmann, K., and du Vigneaud, V., *Science*, **94**, 308 (1941).
8. Fischer, E., and Koeh, H., *Ann. Chem.*, **232**, 227 (1886).
9. Kögl, F., and de Man, T. J., *Z. physiol. Chem.*, **269**, 81 (1941).
10. Thiele, J., *Ann. Chem.*, **308**, 341 (1899).
11. Otto, R., *Ber. chem. Ges.*, **13**, 1272 (1880).
12. Muskat, I. E., and Northrup, H. E., *J. Am. Chem. Soc.*, **52**, 4043 (1930).
13. Przybytek, S., *Ber. chem. Ges.*, **17**, 1092 (1884).
14. Schallt, J., *Wiss. Mitt. österreich. Heilmittelstelle*, **42** (1929), **5** (1930), *Chem. Abst.*, **26**, 4303 (1932).
15. Curtius, T., *J. prakt. Chem.*, **52**, 221 (1895).

THE CONDENSATION OF PHENANTHRENEQUINONE WITH THE DIAMINOCARBOXYLIC ACID DERIVED FROM BIOTIN*

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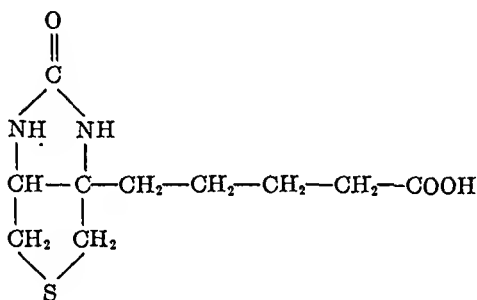
(Received for publication, July 23, 1942)

In a recent communication we reported the preparation of a diamino-carboxylic acid from hiotin (1) and later we reported the resynthesis of hiotin from the diaminocarboxylic acid by treatment of the latter with phosgene (2). This resynthesis confirmed the cyclic urea structure which we had assigned to biotin but did not show whether a 5- or 6-membered urea ring was present. We therefore sought a ring closure for the diamino-carboxylic acid which would distinguish between a 1, 2- and a 1, 3-diamine. This was accomplished by recourse to the formation of a derivative of the diaminocarboxylic acid with phenanthrenequinone. While it is well known that many 1, 2-diamines will condense with phenanthrenequinone, there is no evidence that 1, 3-diamines form a ring structure with this reagent. The diaminocarboxylic acid when treated with phenanthrenequinone yielded a condensation product melting at 202–204° which crystallized in pale yellow needles. The analytical values for the compound agreed somewhat more closely with the composition $C_{23}H_{20}O_2N_2S$ than with $C_{23}H_{22}O_2N_2S$. This together with the fact that a red color was obtained on treatment of the condensation product with sulfuric acid led to the suspicion that we had obtained the quinoxaline rather than the dihydroquinoxaline derivative.

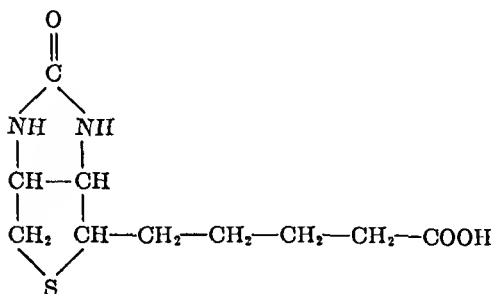
The formation of the derivative ($C_{23}H_{20}O_2N_2S$) with phenanthrenequinone indicated strongly if it did not prove that the diaminocarboxylic acid is a 1, 2-diamine and that therefore hiotin possesses a 5-membered urea ring. This is in contradiction to the suggestion of Kögl and Pons (3), based simply on the comparative stability of 5- and 6-membered cyclic urea derivatives toward hydrolysis, that hiotin is a 6-membered cyclic urea derivative.

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The demonstration of the presence of a 5-membered urea ring eliminates several structures which were previously considered possible in the light of evidence available at that time (4). As we had pointed out, the isolation of adipic acid from the oxidation of the diaminocarboxylic acid and the demonstration that one of the carboxyl groups of adipic acid is identical with the original carboxyl group of biotin were more readily explainable on the basis of a δ -substituted *n*-valeric acid side chain (4). The structures deducible from this interpretation of the data, when taken into consideration with the other known data concerning biotin, led to the suggestion that one of the accompanying formulas (I or II) was the most likely for the structure of biotin.

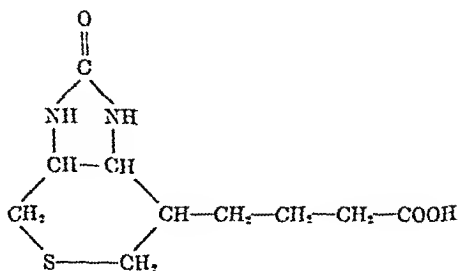


(I)



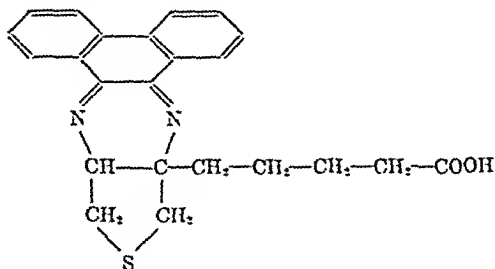
(II)

However, as we pointed out, the formation of the adipic acid might possibly be explained on the basis of the decarboxylation of an intermediary α -substituted β -keto or malonic acid formed during the oxidation procedure (4). If that were the case, a γ -substituted *n*-butyric acid side chain was conceivable and three additional structures came into consideration, two of which contained 6-membered urea rings. The latter two structures are of course eliminated on the basis of the data of the present paper, leaving the structure expressed by Formula III still to be considered as a possibility, although in our estimation this involves a less likely interpretation of the oxidation data.



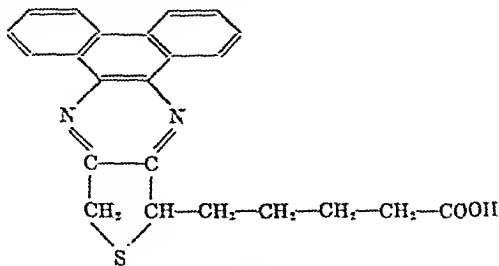
(III)

As indicated earlier, the behavior of the phenanthrenequinone derivative of the diaminocarboxylic acid aroused the suspicion that we may have obtained the quinoxaline rather than the dihydroquinoxaline derivative from the reaction of the phenanthrenequinone with the diaminocarboxylic acid; *e.g.*, Formula I could form a dihydroquinoxaline of the structure expressed by Formula IV but would not be expected to yield the dehy-



(IV)

drogenated derivative. On the other hand the two remaining formulas (II and III) can give the dehydrogenated derivative, since both carbon atoms bearing the amino groups carry hydrogen atoms. For example, Formula II can yield the compound expressed by Formula V. In



(V)

order to settle definitely whether or not the derivative obtained from the diaminocarboxylic acid was the dihydroquinoxaline or the more fully aromatic quinoxaline, we have compared the absorption spectrum of this compound with those of the dibenzodihydroquinoxaline and dibenzoquinoxaline derivatives of 3,4-diaminotetrahydrothiophene.

It would be expected that a great difference in absorption spectra would be shown by the dihydroquinoxaline and quinoxaline forms. As shown in Fig. 1, this expectation is borne out. The absorption spectrum of the di-

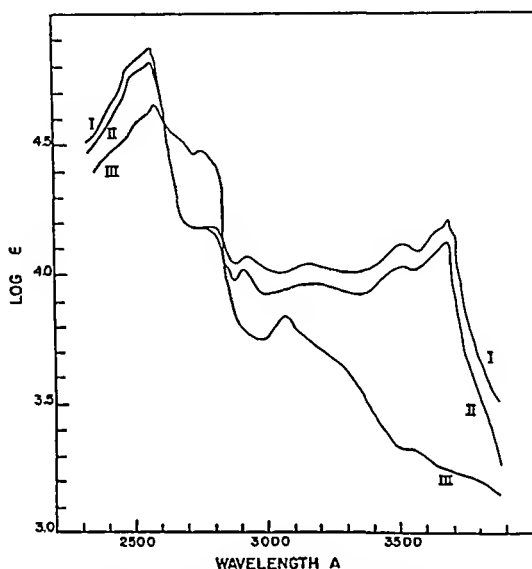


FIG. 1. Ultraviolet absorption spectra of condensation product of phenanthrenequinone with (Curve I) the diaminocarboxylic acid from biotin; (Curve II) 3,4-diaminotetrahydrothiophene, oxidized form; (Curve III) 3,4-diaminotetrahydrothiophene, reduced form.

hydroquinoxaline derivative from the 3,4-diaminotetrahydrothiophene is distinctly different from that of the quinoxaline form of the same compound.

With this demonstration of the striking differences in the absorption curves of the quinoxaline and dihydroquinoxaline derivatives from 3,4-diaminotetrahydrothiophene, it was hoped that the absorption curve of the condensation product of the diaminocarboxylic acid from biotin with phenanthrenequinone would show the characteristics of one or the other of these curves. As shown in Fig. 1, Curve I, the absorption curve of the derivative from biotin is almost identical in form with that of the oxidized, or quinoxaline, derivative from the 3,4-diaminotetrahydrothiophene, and bears little resemblance to the curve of the dihydroquinoxaline derivative.

This is a very strong indication that the derivative formed from phenanthrenequinone and the diaminocarboxylic acid from biotin is a dibenzoquinoxaline, and not a dibenzodihydroquinoxaline, derivative. Thus strong evidence is afforded against Formula I, leaving Formulas II and III still under consideration. As stated earlier (4), "The most logical interpretation" of the adipic acid data "is that biotin contains the side chain, $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{COOH}$, attached to one of the ring carbons." This interpretation of the adipic acid data along with other known facts led to the suggestion of Formulas I and II containing this side chain (4). Since one of these two structures has been eliminated by the data in the present paper, we regard the most likely structure of biotin to be that expressed by Formula II.

EXPERIMENTAL

Condensation of 3,4-Diaminotetrahydrothiophene with Phenanthrenequinone. Dibenzodihydroquinoxaline Derivative—22.2 mg. of 3,4-diaminotetrahydrothiophene (5) were dissolved in 1.5 cc. of methanol and to the solution were added 39 mg. of phenanthrenequinone in 9.5 cc. of ethanol (6). The solution was refluxed for 6.5 hours and was then concentrated *in vacuo* to a small volume. From the cooled solution there were obtained 29.7 mg. of reddish orange prisms, m.p. $171-174^\circ$ when heated rapidly. Concentration of the mother liquors yielded an additional 12.8 mg. The compound was recrystallized from alcohol and from a chloroform-hexane mixture and yielded material with a melting point of $183-185^\circ$. Concentrated sulfuric acid produced a green color with this compound. A solution of the compound in benzene exhibited a dull blue fluorescence.

$\text{C}_{12}\text{H}_{14}\text{N}_2\text{S}$	Calculated.	C 74.45, H 4.86, N 9.65
(290.4)	Found.	" 74.45, " 5.06, " 9.38

Dibenzoquinoxaline Derivative—A sample of the dibenzodihydroquinoxaline derivative was heated at 200° for 10 minutes and then sublimed at 200° and 2 mm. The sublimate was dissolved in the minimum amount of boiling chloroform and the solution was cooled in an ice-salt bath. The material which separated was recrystallized several times from chloroform and chloroform-hexane solution. The unchanged dihydro derivative, which remained in the mother liquors, was resublimed with further conversion to the oxidized form. The pure compound, m.p. $228-233^\circ$, crystallized in pale yellow prisms. A red color was produced with concentrated sulfuric acid. A benzene solution of the compound gave a brilliant blue fluorescence.

$\text{C}_{12}\text{H}_{12}\text{N}_2\text{S}$ (288.4).	Calculated,	C 74.97, H. 4.20; found, C 75.04, H 3.96
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Condensation of Diaminocarboxylic Acid from Biotin with Phenanthrenequinone—14 mg. of the diaminocarboxylic acid sulfate from biotin were converted to the free diaminocarboxylic acid with the calculated amount of $\text{Ba}(\text{OH})_2$ (1). The free diaminocarboxylic acid was dissolved in 7 cc. of ethanol, 10 mg. of phenanthrenequinone were added, and the solution was refluxed for 14 hours. The small amount of insoluble material that had settled out was removed by filtration and the filtrate was then concentrated *in vacuo* to a small volume. Needles separated from the solution after a short time. These were recrystallized twice from an ethanol-water mixture, and yielded 8 mg. of pale yellow needles, m.p. $202-204^\circ$. The compound gave a red color with concentrated sulfuric acid, and in benzene solution showed a strong blue fluorescence.

$\text{C}_{23}\text{H}_{20}\text{O}_2\text{N}_2\text{S}$. Calculated.	C 71.09, H 5.19, N 7.21
(388.45) Found.	" 71.12, " 5.41, " 7.28

Ultraviolet Absorption Spectra—The ultraviolet absorption spectra of the quinoxaline and dihydroquinoxaline derivatives of the 3,4-diaminotetrahydrothiophene and the derivative obtained from the reaction of the diaminocarboxylic acid from biotin with phenanthrenequinone were determined by the use of a Hilger medium quartz spectrograph No. E-3, equipped with a Spekker photometer, and with a special hydrogen discharge tube (7) as the light source. The solvent in all cases was ethanol and the readings were taken at about 21° . The results are shown in Fig. 1 in which the logarithms of the molecular extinction coefficients are plotted against the wave-length.

The authors wish to express their appreciation to Dr. Julian R. Rachele of this laboratory for carrying out the microanalyses.

SUMMARY

The diaminocarboxylic acid derived from biotin by hydrolysis of the urea linkage condenses with phenanthrenequinone to produce a dibenzoquinoxaline derivative, rather than a dibenzodihydroquinoxaline derivative. The structure of the condensation product was determined by analyses, by color reaction with concentrated H_2SO_4 , and by comparison of the ultraviolet absorption spectrum with those of the dibenzoquinoxaline and dibenzodihydroquinoxaline derivatives from 3,4-diaminotetrahydrothiophene.

These results mean in effect that the cyclic urea structure present in biotin is 5-membered, and that the aliphatic acid side chain present in biotin is not attached to either of the carbon atoms bearing the amino groups.

These results in conjunction with our previously published data eliminate several heretofore possible structures for biotin, and leave only two possibilities to be considered, the more likely of which we regard to be that expressed by Formula II.

BIBLIOGRAPHY

1. Hofmann, K., Melville, D. B., and du Vigneaud, V., *J. Biol. Chem.*, **141**, 207 (1941).
2. Melville, D. B., Hofmann, K., and du Vigneaud, V., *Science*, **94**, 308 (1941).
3. Kōgl, F., and Pons, L., *Z. physiol. Chem.*, **269**, 61 (1941).
4. du Vigneaud, V., Hofmann, K., and Melville, D. B., *J. Am. Chem. Soc.*, **64**, 188 (1942).
5. Kilmer, G. W., Armstrong, M. D., Brown, G. B., and du Vigneaud, V., *J. Biol. Chem.*, **145**, 495 (1942).
6. Einhorn, A., and Bull, B. S., *Ann. Chem.*, **295**, 209 (1897).
7. Darby, H. H., *J. Am. Chem. Soc.*, **62**, 1874 (1940).

THE ORGANIC ACIDS OF THE LEAVES OF BRYOPHYLLUM CALYCINUM

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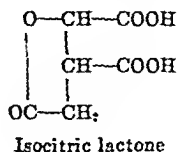
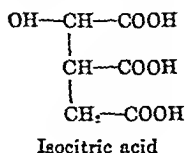
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It has been known since the work of Mayer (1) in 1875 that the thick succulent leaves of many plants of the family Crassulaceae become enriched in titratable organic acids during the night and diminish in acid content when the leaves are illuminated. An analogous diurnal variation in acidity has been observed in a number of other succulents, and occasionally in species of different growth habit (2). This behavior of the organic acids is usually referred to as the crassulacean type of metabolism, and for many years has attracted a great deal of attention.

As a preliminary to a chemical study of the phenomenon, it was necessary to examine the organic acids of a typical species in some detail, particularly since modern methods for the isolation of organic acids have hitherto been applied in only one case (3) and, as will be made clear in another connection (4), there is reason to be dissatisfied with the published results.

The organic acids have accordingly been isolated from the leaves of *Bryophyllum calycinum*, the species originally examined by Mayer, and separated by means of distillation of the ethyl esters. Indirect analysis of a sample of the leaves of this common greenhouse plant showed that, in addition to small proportions of oxalic and succinic acids, roughly 7 per cent of the dry weight consisted of *l*-malic acid, 2 per cent of citric acid, and 14 per cent of "unknown acids" (the total organic acidity minus the sum of the acidity due to malic, citric, and oxalic acids) calculated arbitrarily as citric acid. The present study has shown that by far the greater part of the "unknown acids" consists of *isocitric acid*.

The bearing of this wholly unexpected observation upon the old and puzzling problem of the nature of the so called crassulacean malic acid is discussed in another paper (4). For the present, it is desirable to point out that *isocitric acid*, although synthesized many years ago by Fittig and



Miller (5), and by Wislicenus and Nassauer (6), was found in nature for the first time in 1925 by Nelson (7, 8) in the juice of blackberries. It is the major organic acid component of this fruit. Nelson's observation has been confirmed by Bruce (9) who has also provided helpful information on the chemical behavior of isocitric acid and of its lactone. More recently, the substance has been detected in minor amounts in several other species by Nelson and his collaborators (10).

Franzen and Keyssner (11), in 1923, carried out a careful study of the leaves of the blackberry plant and record the isolation, from the esters of high boiling point, of an unidentified hydrazide that decomposed at 181–182° and contained 34.6 per cent of nitrogen. In spite of the low nitrogen content (theory 35.9 per cent), Nelson has pointed out that this may well have been a specimen of the trihydrazide of isocitric acid; owing to Franzen's death before the work was published, no further investigation of the compound was made.

Although isocitric acid is the dominant organic acid of blackberry fruit, the proportion present is relatively small. Nelson secured about 141 gm. of ester from extracts that represented 54 kilos of fresh fruit or 8.9 kilos of dry weight; this is the equivalent of 0.18 per cent of the fresh weight or 1.1 per cent of the dry weight of the tissue. As a minimum, the yield of isocitric acid from *Bryophyllum* leaves has been found to be 8.1 per cent of the dry weight, this being the proportion secured as distilled esters; the actual proportion present was doubtless considerably larger. The leaves of *Bryophyllum* are accordingly an unusually rich source of this rare acid.

EXPERIMENTAL

Extraction of Leaf Tissue—Several lots of dry powdered leaf tissue were combined for the isolation of the organic acids. Analyses of two of these lots, shown in Table I, illustrate the high relative proportion of "unknown" organic acids in both, and the wide variation in *l*-malic acid content, a phenomenon which is probably connected with the time of day the leaves were collected. Extracts were prepared from 427 and from 500 gm., respectively, of the two lots of dry tissue by stirring the powder for half an hour in each case with 5 times its weight of water at a temperature of 60–65°. The temperature was chosen so as to bring about as little hydrolysis of pectins as possible. The tissue was strained off on cheese-cloth and pressed, and the residue was extracted twice more in the same way. Analysis showed that 97 per cent of the malic acid, 99 per cent of the citric acid, and 84 per cent of the unknown acids had been extracted.

The extracts were each concentrated *in vacuo* to about 500 ml., and were brought to pH 1.0 by the addition of the requisite amount of sulfuric acid and treated with 2 volumes of alcohol. The precipitate, which consisted

mainly of inorganic salts, was centrifuged off and washed with 60 per cent alcohol three times, and the alcoholic solution was concentrated to about 1 liter. To this, hot saturated barium hydroxide solution was added to pH 9 to 10, followed by 2 volumes of alcohol, and the precipitate was allowed to settle overnight. The clear fluid was decanted; the barium salts were centrifuged and washed three times with 60 per cent alcohol, and were then suspended in 3 liters of water, thoroughly stirred, and again treated with 2 volumes of alcohol. The reprecipitated barium salts were centrifuged off, washed twice with 60 per cent alcohol, suspended in warm water, and treated with a slight excess of sulfuric acid. The barium sulfate was centrifuged, washed repeatedly with hot water, and the solution was concentrated to about 150 ml.

TABLE I

Organic Acids of Leaf Tissue of Bryophyllum calycinum

Sample 1939-C was a mixture of two collections of leaves, one of which was taken at 9 a. m., the other at 2 p. m. on different dates. The plants were grown in sand with a complete nutrient solution that supplied nitrogen as nitrate. Sample 1940-D comprised leaves collected in the afternoon from plants grown in soil. All leaves were promptly dried in a current of air at 80°. For methods of analysis see (12).

Figures not otherwise designated are milliequivalents per 100 gm. of dry tissue.

	Sample 1939 C	Sample 1940-D
Total organic acids	427	301
<i>l</i> -Malic acid	166	51.1
Citric "	42.4	21.7
Oxalic "	5.9	5.9
Unknown acids (by difference)	212.7	222.3
" " % total acids	49.1	73.9
Dry weight of leaves, % fresh weight	7.3	7.8

The organic acids were extracted from this solution with ether in an apparatus of the Widmark type (13). The extraction period was 120 hours in one case and 168 hours in the second; 8 N sodium hydroxide was used to collect the organic acids, being renewed when it became neutral to phenolphthalein. The solution of the sodium salts obtained was diluted to 500 ml., brought to pH 1.0 with sulfuric acid, and was then treated with 2 volumes of alcohol. After the solution had been chilled overnight, the sodium sulfate was filtered off, washed thoroughly with 70 per cent alcohol, and the alcohol solution of the free organic acids was concentrated to a sirup which was repeatedly dehydrated by the addition of absolute alcohol followed by distillation. At this point, the solutions from the two lots were combined. The acids were esterified by the method of Phelps and Phelps (14), the crude esters were taken up in ether, and, in order to reduce as

much as possible the loss due to solubility of the esters in water, were washed with a 20 per cent sodium sulfate solution to which sufficient 20 per cent sodium carbonate was added to make the aqueous phase permanently alkaline to litmus after having been shaken with the ether. The aqueous layer was drawn off, the layer of emulsion was centrifuged, again separated, and the collected ether extracts were washed with small portions of 20 per cent sodium sulfate solution until neutral. The ether solution was then dried over anhydrous sodium sulfate and the ether was distilled.

From 927 gm. of dry tissue, 220 gm. of crude esters were secured of which 209 gm. remained after the ether solution had been washed with alkaline

TABLE II
Fractional Distillation of Esters of Organic Acids of Bryophyllum calycinum Leaves at 3 Mm. Pressure

Fraction No.	B.p.	Weight	Specific rotation, $[\alpha]_D^{25}$	Composition of fraction		
				Diethyl malate	Triethyl citrate	Unknown
	°C.	gm.	degrees	per cent	per cent	per cent
1	97-102	4.7	-10.0			
2	102-107	64.8	-10.75	100	0	0
3	107-108	2.97		73.4	5.0	21.6
4	108-128	2.09		68.8	6.8	24.4
5	128-137	0.95		40.7	14.1	45.2
6	137-145	11.1	-7.13	0.0	28.2	71.8
7	145-151	20.4	-14.03	0.0	26.8	73.2
8	151-156	18.9	-26.02	0.0	20.2	79.8
9	156-160	28.5	-42.6	0.0	8.9	91.1
10	160-161	34.8	-53.3	0.0	0.7	99.3
Residue		10.0				
Total		199.2				

sodium sulfate solution. The esters were distilled through a column filled with beads and equipped with a dephlegmator, and were separated into fractions as shown in Table II. The fractions, together with an undistilled residue of 10 gm., account for 95.6 per cent of the weight of the washed esters. The composition of the fractions was ascertained by analysis of small samples after saponification, and by examination of the hydrazides.

Fraction 1, from its specific rotation, was nearly pure *l*-malic ester, and the hydrazide, which melted at 179-180°, was apparently pure. Although traces of oxalic and succinic esters were doubtless present, no detailed examination of the fraction was made for them. Both of these acids are present in small amounts in *Bryophyllum* leaf tissue (15). Fraction 2

was pure *l*-malic diethyl ester; the specific rotation agrees with earlier observations on material prepared from tobacco leaves (16). *Fractions 3, 4, and 5* were small intermediate fractions that contained both malic and citric esters together with a regularly increasing relative proportion of ester of unknown acid. They were not further studied.

The regular decrease in citric acid in the three following fractions, together with the regular increase in specific rotation and in the proportion of unknown acid, turned attention to *Fraction 10*, which gave evidence, from the constant boiling point, of being substantially a pure substance. The high specific rotation in particular recalled the properties of isocitric acid, since Bruce (9) has shown that the specific rotation of diethyl isocitrate lactone is $[\alpha]_{589}^{26} = -54.2^\circ$, and the esters of no other well known organic acids are so strongly levorotatory as this. Analysis of a sample of the ester gave C 51.89, 51.95; H 6.37, 6.26 per cent; the theory for $C_{10}H_{14}O_6$ (diethyl isocitrate lactone) is C 52.17, H 6.13; while that for triethyl isocitrate is C 52.15, H 7.30 per cent. These observations strongly suggest that *Fraction 10* consisted largely of the diethyl ester of isocitric lactone.

Identification of Isocitric Acid—A sample of 1.40 gm. of *Fraction 10*, dissolved in 8 ml. of alcohol, was treated with an excess of hydrazine hydrate: the solution remained clear for about 30 seconds, when crystallization of a white substance suddenly began. The crystals were filtered, after the solution had been chilled for a few hours, were washed with absolute alcohol and ether, and dried at 105° ; yield 1.28 gm., or 104 per cent, calculated as dihydrazide, 89.4 per cent calculated as trihydrazide. The product melted at $178-179^\circ$ with decomposition, but gave a marked depression of the decomposition point when mixed with pure *l*-malic dihydrazide. This decomposition point is identical with that observed by Nelson for a similar crude product prepared from the isocitric acid of blackberries. On recrystallization from 60 per cent alcohol, the decomposition point was raised to $181-182^\circ$. Elementary analyses are shown in Table III which indicate that the substance was the trihydrazide of isocitric acid. The ring is evidently opened during the formation of the hydrazide from the ester of the lactone.

Isocitric Trihydrazide—This substance is not entirely satisfactory for the certain identification of isocitric acid. It separates from alcohol or dioxane solutions sometimes in sheaves of feathery needles, and sometimes in fine hair-like needles which form a firm mat on the filter paper, but more often in rosettes of short needles. The crystals are fragile and must be examined under the microscope before filtration. No correlation between crystalline habit and decomposition point could be established.

The decomposition point of the preparations from the crude ester was usually within a degree or two above or below 181° ; on recrystallization the

decomposition point may remain constant at or near 181° , or may rise to 185° . One preparation was obtained which, after one recrystallization, decomposed at $195\text{--}196^{\circ}$ but, after further recrystallization, the decomposition point dropped to $188\text{--}190^{\circ}$. Nelson (8) records one preparation that decomposed at $196\text{--}197^{\circ}$ and concluded, from the optical crystallographic data and comparison with synthetic material, that the product from blackberries was partially racemized. However, Bruce secured a preparation from optically active material that decomposed at $201\text{--}202^{\circ}$. It was noted in the present work that the yields after recrystallization were low, and that the mother liquors, on evaporation, deposited manifestly impure material. The substance is apparently not entirely stable during ordinary manipulations, and it is quite likely that completely homogeneous material was not

TABLE III
Analyses of Isocitric Acid Trihydrazide

Found*		Calculated	
		Isocitric trihydrazide, $C_8H_{10}N_6O_6$	Isocitric lactone dihydrazide, $C_8H_{10}N_4O_4$
	per cent	per cent	per cent
C.....	30.80, 30.95	30.77	35.63
H.....	6.06, 5.98	5.98	4.95
N.....	34.84, 34.89	35.90	27.72

* Grateful acknowledgment is made to Mr. William Saschek of the Department of Biochemistry of the College of Physicians and Surgeons, Columbia University, New York, for these and the other elementary analyses in this paper. Nitrogen determinations on hydrazides, even by the micromodification of the Dumas method, are usually low.

secured. The benzylidene derivative of the trihydrazide is not a satisfactory product for confirmation of the identity, since it is insufficiently soluble in the usual solvents to be readily recrystallized. An amorphous product of decomposition point $193\text{--}197^{\circ}$ was secured.

Isocitric Lactone and Derivatives—A 5 gm. sample of Fraction 10 was hydrolyzed by being boiled with 1 N hydrochloric acid, and the acid was esterified with methyl alcohol. The dimethyl ester of the lactone distilled completely between $136\text{--}138^{\circ}$ at 2 mm. pressure, and it was necessary to warm the condenser to prevent crystallization of the distillate. The distillate solidified to a colorless crystalline mass that melted at $106\text{--}107^{\circ}$. Recrystallized from methyl alcohol, the substance melted at $107.5\text{--}108^{\circ}$ uncorrected ($108.5\text{--}109^{\circ}$ corrected). Bruce observed a melting point of $105\text{--}106^{\circ}$. Analysis of the substance gave C 47.74, 47.75; H 5.08, 5.00; theory for $C_8H_{10}O_6$ (dimethyl isocitrate lactone) C 47.52, H 4.98 per cent. The

trihydrazide prepared from this material decomposed at 184–185° and gave C 30.75, H 6.03 per cent, in almost exact agreement with theory. These data complete the identification of the predominant acid of *Bryophyllum* leaves as isocitric acid.

A further specimen of Fraction 10 was saponified with barium hydroxide and the precipitated barium salt was filtered from the boiling solution. The free acid was liberated with sulfuric acid, the excess of which was quantitatively removed, and the solution was evaporated to a sirup that was heated *in vacuo* at 100° for several hours, and was then kept in an evacuated desiccator over phosphorus pentoxide for a month, when it had solidified. The mass was dissolved in ethyl acetate and allowed to crystallize by spontaneous evaporation. The white hygroscopic crystals, on being heated, softened at 152° and melted at 153–154°. Bruce records the melting point of isocitric lactone as 154°. Of this product, 0.058 gm. (equivalent to 0.064 gm. of isocitric acid) required 1.33 ml. of 0.508 *N* sodium hydroxide for neutralization at room temperature and 1.95 ml. to reach a permanent end-point after the solution was heated to boiling temperature for 20 minutes. These quantities are equivalent, respectively, to 0.0588 gm. of lactone and 0.0634 gm. of free acid.

Fractions 6, 7, 8, and 9—The identification of diethyl isocitrate lactone as the chief component of Fraction 10 permitted an estimate of the composition of these intermediate fractions to be made. On the assumption that the optical activity arose from the presence of the ethyl ester of the lactone, the fractions were calculated to contain respectively 13, 26, 48, and 78 per cent of isocitric acid. Isolation of the trihydrazides, however, gave evidence of the presence of much higher proportions of isocitric acid than this, particularly in Fractions 6 and 7. Accordingly, in addition to moderate quantities of triethyl citrate, it seemed probable that these fractions contained both triethyl isocitrate and the ester of isocitric lactone.

No procedure was found whereby saponification of the ester could be effected without partial or complete opening of the lactone ring. After being heated with 1 *N* hydrochloric acid, specimens of these fractions became weakly dextrorotatory, indicating hydrolysis to the acid. When aqueous solutions of the acid freed from hydrochloric acid (pH 1.7) were evaporated to sirups and heated on the steam bath, the rotation changed to the left, indicating partial conversion to the lactone, the solutions being the more strongly levorotatory the more intense the dehydrating conditions that were applied. Dilute aqueous solutions of these levorotatory mixtures of acid and lactone, when heated at 100°, gradually became inactive or even weakly dextrorotatory owing to more or less complete conversion back to the acid. These observations provide an exact parallel to the confusing behavior recorded without explanation by Aberson (17) in connection with an

acid fraction obtained from the leaves of another species of Crassulaceae (see (4) for a discussion), and clearly indicate the desirability of a thorough physicochemical study of the equilibrium between isocitric acid and its lactone.

Information on the composition of the fractions, with respect to isocitric acid, was obtained by subjecting samples to complete hydrolysis and studying the rotation in the presence of molybdenum salts according to the method outlined by Martius (18). This procedure is based upon the assumption that no optically active substance other than isocitric acid is present. The fundamental observation is the specific rotation of pure isocitric acid in the presence of ammonium molybdate. To obtain this,

TABLE IV

Specific Rotation of Isocitric Acid in Presence of Ammonium Molybdate

The lactone or its ester was hydrolyzed with a small excess of sodium hydroxide on the steam bath, the sample was cooled, acidified with 0.5 ml. of glacial acetic acid, and 2 gm. of ammonium molybdate were added. The solution was diluted to 15, or to 20, ml. and the rotation was observed with a Ventzke sugar scale instrument with incandescent electric light and converted to degrees of circular rotation by the factor 0.3468.

Sample	Weight	Equivalent of isocitric acid	Specific rotation, $[\alpha]^{25}$	Hydrolysis time
	gm.	gm.	degrees	min.
Isocitric lactone	0.0609	0.0672	-370	10
	0.0580	0.0640	-368	30
Dimethyl isocitrate lactone	0.1722	0.1636	-358	90
	0.1722	0.1636	-358	90
	0.1722	0.1636	-362	180
Average			-363	
Isocitric lactone	0.0699		-69.6	0

the purest available specimens of the lactone and of its dimethyl ester were saponified with a slight excess of alkali at boiling temperature, and were then acidified with acetic acid and treated with an excess of ammonium molybdate. Data from a series of such experiments are shown in Table IV; the average value of the specific rotation of the molybdenum complex was -363° . This figure is appreciably lower than the value -413° obtained by Martius for isocitric acid prepared from aconitic acid by the action of an enzyme present in liver extract. His result, however, rests upon the examination of a single specimen. It is to be noted that the specific rotation of the lactone itself is little, if at all, increased under these conditions.

The specific rotation in the presence of molybdate ion of completely

hydrolyzed samples of the ester fractions permitted calculation of the proportion of isocitric acid in each; these results are shown in Column 2 of Table V. If Fraction 10 consisted of pure diethyl ester of isocitric lactone, the isocitric acid content would theoretically be 83.4 per cent. From the specific rotation data, its purity with respect to isocitric acid was thus 91 per cent.

An estimate of the composition of the intermediate fractions of esters was made from these results for the isocitric acid content in combination with the specific rotations of the individual fractions shown in Table II. If it be assumed that the only optically active substances present are the triethyl ester of isocitric acid and the diethyl ester of its lactone, it is possible to calculate from the specific rotations in the literature, which are, respectively $+14^\circ$ (7)¹ and -54.2° (9), the relative proportions of these in each fraction. From this, in turn, the relative percentage composition

TABLE V
Estimated Composition of Ester Fractions 6 to 10 (Table II)

Fraction No (1)	Isocitric acid content (2)	Relative composition of fractions			
		Triethyl isocitrate (3)	Diethyl isocitrate lactone (4)	Triethyl citrate (5)	Unknown (6)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
6	43.4	33.5	25	28.2	13.3
7	56.9	35	39	26.8	-0.8
8	60.1	19.3	56.5	20.2	4
9	72.3	6	82	8.9	3.1
10	76.0	0	91	0.7	8.3

shown in Columns 3 to 6 of Table V can be computed. The figures in Column 5 are derived from analytical determinations of citric acid in each fraction, and are presumably accurate. The results for the esters of isocitric acid are at best rough approximations.

The chief object of these estimates is to afford a demonstration of the complexity of the intermediate fractions. Although they become successively richer in isocitric acid and poorer in citric acid, it is obvious that only an elaborate fractional distillation process with a far more efficient apparatus than was used in these experiments would bring about sharp separations of the components. Column 6 suggests that contamination with organic acid esters other than those of citric and isocitric acids was practically negligible.

¹ An ester of specific rotation $+14^\circ$ was obtained by Nelson in a fraction of high boiling point. It seems probable that this fraction was exceptionally rich in triethyl citrate, although Nelson makes no claim that it was a pure specimen.

Inorganic Salts of Isocitric Acid—Although the literature of isocitric acid contains many references to salts of isocitric acid with calcium, barium, and silver, these substances have not been recorded as being prepared in crystalline form, and analytical results upon the preparations are rarely in good agreement with the requirements of theory. The experience with the material derived from *Bryophyllum* leaves was similar. The barium salt was prepared from samples of Fraction 10 after saponification with barium hydroxide, or sulfuric acid, and was also prepared from the purest available specimen of the dimethyl ester of the lactone. It was established that the salt, after being dried at 105°, was anhydrous, since no further loss in weight occurred when it was heated to 150°. Nevertheless the preparations invariably contained from 51.2 to 51.5 per cent of barium instead of the theoretical 52.16 per cent. All were amorphous powders. Products prepared by precipitation from aqueous solution with alcohol gave erratic analytical results, and no improvement was obtained by attempts to recrystallize the material from hot water.

The calcium salt is even less satisfactory. It is conveniently obtained by heating a cold aqueous solution to the boiling point. Such products are hydrates and are amorphous. Two preparations, separated from slightly alkaline solution, contained an average of 14.15 per cent of water of hydration, a proportion that is not related in any simple way to the composition of possible hydrates of calcium isocitrate. The calcium content of the anhydrous material was, respectively, 21.8 and 22.0 per cent; theory for tricalcium isocitrate 24.12 per cent.

The behavior of the calcium salt provides an interesting, although of course by no means specific, diagnostic test for isocitric acid. A solution that contains up to 2 per cent of the salt can readily be obtained in the cold and may be evaporated at low temperature to about one-third of its volume without precipitation. When heated, however, the salt separates as a curdy precipitate in a manner that recalls the heat coagulation of a protein. A 0.5 per cent solution of the calcium salt likewise gives a heavy precipitate when boiled, but in this case the precipitate soon redissolves when the solution is cooled.

Conditions have not been found under which silver salts that yield interpretable analytical results can be prepared.

General Properties of Isocitric Acid—The most useful compound of isocitric acid for identification purposes that has been studied is the dimethyl ester of the lactone. This crystallizes well and can be characterized by its melting point and other physical properties, as well as by its qualitative behavior, with little danger of error. Owing to its apparent instability on recrystallization, the trihydrazide is less useful, and confusion of the decomposition point with that of malic acid dihydrazide is quite possible.

Isocitric acid does not give the Denigès reaction characteristic of citric acid. This test may accordingly be applied to detect contamination of preparations with citric acid. Isocitric acid does not yield pentabromoacetone when oxidized with bromine and permanganate (Stahre reaction) as does citric acid. Furthermore, no halogen-containing compound soluble in petroleum ether is formed during this oxidation, and no color is formed when the petroleum ether extract of the oxidation mixture is treated with sodium sulfide. Accordingly, isocitric acid does not interfere with the determination of citric acid by the methods customarily employed in this laboratory (12, 19).

When isocitric acid is oxidized with bromine and permanganate and the clarified solution is heated with dinitrophenylhydrazine, a yellow product is precipitated. The solution of this in pyridine becomes reddish brown when a slight excess of sodium hydroxide is added. However, the oxidation product of isocitric acid is not volatile with steam and, accordingly, does

TABLE VI
Distribution of Major Organic Acid Components of Bryophyllum Leaves

Acids	Calculated from composition of esters	Calculated from composition of water extract
	<i>per cent</i>	<i>per cent</i>
Malic .	36.1	33.2
Citric	7.8	9.2
Isocitric	52.2	
Unknown*	3.9	57.6

* Includes the small proportion of oxalic and succinic acids known to be present.

not interfere with the method for the determination of malic acid, which depends upon distillation of the oxidation product, conversion to the dinitrophenylhydrazone, and colorimetric determination of the blue compound formed when this is treated in pyridine solution with alkali (12).

Isocitric Acid in Bryophyllum Leaves—From the data in Table I it can be calculated that the 927 gm. of dry leaf tissue employed for the isolation of the organic acids contained 6.9 per cent of malic acid, 2.0 per cent of citric acid, and 13.9 per cent of unknown acid if this is assumed to have been a tribasic organic acid of the same molecular weight as isocitric acid. Analysis of the water extract from this sample of tissue indicated the presence of the equivalent of 6.8 per cent of malic acid, 1.9 per cent of citric acid, and 11.8 per cent of unknown acid when expressed in the same terms and on the same assumption. Almost exactly three-quarters of the organic acids of the water extract was obtained as esters and subjected to distillation. From the data on the composition of the ester fractions in Table II

the distribution of the three main acids in the esterified material can be computed, and these values are compared in Table VI with the distribution of the organic acidity in the water extract of the leaves. The close resemblance suggests that the material secured in ester form represented a fair sample of the organic acids in the water extract of the tissues. It may be concluded, therefore, that a determination of the "unknown" organic acids of *Bryophyllum* leaves by the analytical methods now in use gives a reasonably close estimate of the proportion of isocitric acid present. A figure so obtained would probably be not more than 10 per cent of itself too high, and could be made somewhat more accurate by the determination of oxalic and succinic acids in the sample. This conclusion is useful at the present time, since a specific analytical method for the determination of isocitric acid in plant tissues has not yet been developed.²

DISCUSSION

Isocitric acid is the predominant organic acid of the leaf tissue of *Bryophyllum calycinum* and, as will be made clear in another paper (4), was doubtless the main component of the preparations obtained by early workers that were regarded as specimens of a new and different form of malic acid. According to the older literature, so called crassulacean malic acid is widely distributed in the group of plants to which this species belongs. The observation in recent years that isocitric acid occurs, although only in small proportions, in a number of unrelated plants suggests that, if suitable analytical methods are developed, the substance will be found to be a not uncommon constituent of plants and possibly of animal tissues as well.

Present day speculations on the oxidative mechanisms in the animal body, as well as on the mechanisms that have to do with the assimilation of carbon dioxide in plants, lay much stress upon schemes of enzymatic reactions in which a considerable variety of organic acids is concerned. Most, if not all of these organic acids are well known constituents of plant tissues; some of them are present in certain plants in astonishingly high concentrations. The present case is especially noteworthy, since it emphasizes the significance of the rôle that isocitric acid, a substance hitherto rarely encountered in nature, may play.

SUMMARY

An examination of the organic acids extracted by warm water from dried leaves of *Bryophyllum calycinum* has shown that the sample studied con-

² Methods based upon the specific rotation in the presence of uranium or molybdenum salts are inapplicable in the presence of malic acid, and the only reliable method available for the separation of malic and isocitric acids is the fractional distillation of the esters.

tained at least 8 per cent of the dry weight, and probably appreciably more, of isocitric acid together with about 7 per cent of *l*-malic acid and 2 per cent of citric acid. This plant is thus by far the richest source of isocitric acid that has hitherto been encountered. Attention is briefly drawn to the theoretical implications of this observation.

BIBLIOGRAPHY

1. Mayer, A., *Landw. Versuch.-Stat.*, 18, 410 (1875); 21, 277 (1878); 34, 127 (1887).
2. Bennet-Clark, T. A., *New Phytologist*, 32, 39, 123, 197 (1933).
3. Franzen, H., and Ostertag, R., *Z. physiol. Chem.*, 122, 263 (1922).
4. Pucher, G. W., and Vickery, H. B., *J. Biol. Chem.*, 145, 525 (1942).
5. Fittig, R., and Miller, H. E., *Ann. Chem.*, 255, 43 (1889).
6. Wislicenus, W., and Nassauer, M., *Ann. Chem.*, 285, 1 (1895).
7. Nelson, E. K., *J. Am. Chem. Soc.*, 47, 568 (1925).
8. Nelson, E. K., *J. Am. Chem. Soc.*, 52, 2928 (1930).
9. Bruce, W. F., *J. Am. Chem. Soc.*, 57, 1725 (1935).
10. Nelson, E. K., and Wheeler, D. H., *J. Am. Chem. Soc.*, 59, 2499 (1937). Curl, A. L., and Nelson, E. K., *Am. Potato J.*, 17, 328 (1940).
11. Franzen, H., and Keyssner, E., *Z. physiol. Chem.*, 129, 309 (1923).
12. Pucher, G. W., Wakeman, A. J., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, 13, 244 (1941).
13. Pucher, G. W., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, 11, 656 (1939).
14. Phelps, I. K., and Phelps, M. A., *Am. J. Sc.*, 174, 194 (1907).
15. Pucher, G. W., and Vickery, H. B., *Ind. and Eng. Chem., Anal. Ed.*, 13, 412 (1941).
16. Vickery, H. B., and Pucher, G. W., *Connecticut Agric. Exp. Stat., Bull.* 523 (1931).
17. Abernethy, J. H., *Ber. chem. Ges.*, 31, 1432 (1898).
18. Martius, C., *Z. physiol. Chem.*, 257, 29 (1938).
19. Pucher, G. W., Vickery, H. B., and Leavenworth, C. S., *Ind. and Eng. Chem., Anal. Ed.*, 6, 190 (1934). Pucher, G. W., Sherman, C. C., and Vickery, H. B., *J. Biol. Chem.*, 113, 235 (1936).

ON THE IDENTITY OF THE SO CALLED CRASSULACEAN MALIC ACID WITH ISOCITRIC ACID

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Malic acid is one of the most widely distributed organic acids found in plant tissues. The literature to 1923 has been carefully reviewed by Franzen and Keyssner (1) and, although these investigators pointed out that the evidence that has been advanced for the identity of the substance isolated has in only a few cases been completely convincing, a study of their paper shows that serious doubt can seldom be entertained that the preparations described contained malic acid. Many new records of the occurrence of malic acid have since been made. However, there is one group of observations that has proved puzzling to both chemists and plant physiologists for more than 60 years. In 1875, Mayer (2) noted that leaves of *Bryophyllum calycinum*, a species in the family Crassulaceae, collected in the early morning, contain a considerable proportion of titratable free acid, and that the acidity diminishes after the leaves are exposed to light for a few hours. This observation was even then by no means new, for a marked difference in the taste of the leaves at different times of day had been recorded many years before. Mayer put the matter on a quantitative footing and, in addition, noted that the disappearance of the acid was accompanied by an evolution of oxygen which took place even when carbon dioxide was excluded from the air surrounding the leaves. The observations were extended to a number of allied species and evidence was obtained (3, 4) that the evolution of oxygen and the disappearance of acid were closely connected phenomena.

An examination of the organic acids separated as calcium salts from extracts of *Bryophyllum* leaves gave evidence for the presence of a substance, the salts of which corresponded closely in composition to those of malic acid. The physical properties both of the salts and of the free acid were, however, different from those of *l*-malic acid, and Mayer inferred that the substance prepared from these leaves was an isomer.

This substance has passed into the literature under the name "crassulacean malic acid," or sometimes as "crassulaic acid," and has been studied by several investigators (for a review see Bennet-Clark (5)). Schmidt (6), for example, prepared ordinary crystalline calcium malate from

Bryophyllum leaves collected during the night, but could obtain only the amorphous salt described by Mayer from leaves collected during the day.

In 1898, Aberson (7) pointed out that Mayer's acid constituted an exception to the rules of stereoisomerism. No structure that contains an asymmetric carbon atom could be assigned save that of malic acid, and the three possible optical isomers of this substance were known and were different from crassulacean malic acid. After an extremely careful and thorough investigation of the substance, Aberson postulated that the isomerism arose from an internal constraint which prevented free rotation about the bond between the central carbon atoms, and provided evidence sufficiently impressive to elicit a discussion of this unique anomaly from Werner (8) in his text-book on stereochemistry (see also Walden (9)).

Work of Franzen and Ostertag—In 1922, Franzen and Ostertag (10) pointed out that the isolation methods employed by the earlier workers—chiefly precipitation of the calcium salts by means of alcohol—were not capable of yielding adequately pure products, and that the alleged differences between crassulacean malic acid and *l*-malic acid might well be accounted for by the presence of impurities in the preparations. They emphasized the fact that no crystalline salts or derivatives of the acid had been described. Walden (9) had shown that malic acid is, in part, converted into a mixture of anhydrides when heated to 100° or higher, and that contamination of a preparation with these products renders crystallization of the remaining malic acid difficult or impossible. They concluded, accordingly, that the preparations of crassulacean malic acid described by earlier workers, especially by Aberson, were mixtures. In support of this contention, they prepared the organic acids from a large sample of leaves of *Echeveria secunda glauca*, separated the individual acids by distillation of the ethyl esters, and showed that the greater part of the acids of this plant does indeed consist of *l*-malic acid, the ester of which distills in the neighborhood of 128° at 11 mm. pressure. A second fraction that distilled at about 180° at the same pressure yielded a dihydrazide that was identified by melting point and mixed melting point, as well as by nitrogen content, as malic dihydrazide, and a sample of this ester, after saponification, yielded a silver salt that seemed identical with preparations made from malic acid. Franzen and Ostertag concluded that the substance of high boiling point consisted largely of the diethyl ester of the cyclic anhydride of malic acid, that is a substance of the type described by Walden, and that there was no evidence to support the view that the tissues of plants of the family Crassulaceae contain a unique form of malic acid.

The latter part of this conclusion was indeed literally correct and the evidence in support of it seemed conclusive. Unfortunately the statement has had the effect of turning attention away from the chemical problems

raised by the curious behavior of the organic acids of the leaves of these plants and has been accepted as the complete explanation of the stereochemical anomaly discussed by Aberson and by Werner.

In order to accept Franzen and Ostertag's interpretation of their own results, it is necessary to ignore a great deal of careful experimental work. Mayer's extensive data cannot be lightly dismissed and, whether Mayer's and Aberson's specimens of crassulacean malic acid were contaminated or not, they were *dextrorotatory*. Natural malic acid is weakly *levorotatory* and its anhydrides, prepared by heating the free acid, are only moderately strongly *levorotatory* (9), and yield malic acid of the original rotation when saponified. Furthermore, a solution of the neutral calcium salt of *l*-malic acid deposits the salt in crystalline form when heated and the crystals remain insoluble when the suspension is cooled. Both Mayer and Aberson found that a solution of the calcium salt of crassulacean malic acid when heated deposits the salt in amorphous form and that the salt promptly redissolves when the solution is cooled (3).

The weakness of Franzen and Ostertag's position is also suggested by a careful examination of their own evidence. They worked with only one sample of a single species (*Echeveria*) instead of with several species as did both Mayer and Aberson, but extended their conclusions to cover the entire problem; they preferred to ignore the fact that their acid prepared from *Echeveria* leaves gave precipitation reactions different from those of authentic malic acid; moreover, they appear to have paid little attention to the fact that the behavior noted during the crystallization of the hydrazide secured from their ester fractions of high boiling point was unusual and quite different from that of malic dihydrazide. The most convincing evidence they adduce for the view that their higher fractions indeed consisted mainly of compounds of ordinary malic acid was the melting point of the hydrazide and of its benzylidene derivative, together with the unsupported statement that these melting points were unchanged when the preparations were mixed, respectively, with authentic malic dihydrazide or with its benzylidene derivative.

This evidence amounts, then, merely to a demonstration of the probability that their ester fraction of high boiling point contained malic acid; it does not preclude the presence of other acids. Furthermore, since the decomposition point of malic acid dihydrazide is 178–179°, while that of isocitric acid trihydrazide is usually not far from 181°, the evidence of the mixed melting points becomes of paramount importance, and these data were not given.

Identity of Crassulacean Malic Acid—The isolation of substantial amounts of isocitric acid from the leaves of *Bryophyllum calycinum* (11) raised the question whether the early investigators of crassulacean malic

acid may have had preparations of this substance in hand without recognizing their identity. The outstanding properties upon which a judgment may be based are the specific rotation of the acid and its derivatives and the behavior of the calcium salts. Other criteria are the composition of the salts and the failure of most salts and derivatives of isocitric acid to crystallize, in contrast to those of *l*-malic acid.

Mayer's (3) original preparation was admittedly impure, but it was dextrorotatory and he stated clearly that the amorphous calcium salt differed in properties from those of either citric acid or malic acid. The most striking point is that this salt was insoluble in hot water but redissolved in the cold and contained 22.24 per cent of calcium. Authentic specimens of calcium isocitrate usually contain about 22 per cent of calcium, appreciably lower than the theoretical 24.1 per cent, and their behavior in hot and cold water is identical with that described by Mayer. The evidence is inconclusive, but it is by no means impossible that Mayer's material contained a considerable proportion of isocitric acid.

Aberson's careful description of his products leaves little question that he had fairly pure specimens of isocitric acid or derivatives of this in hand. His method of isolation by means of successive precipitation of the lead and calcium salts could scarcely be expected to lead directly to analytically pure specimens, and the data shown in Table I for the silver and lead salts prepared from material thus secured clearly agree somewhat more closely with the theoretical requirements for malic acid than for isocitric acid. It is obvious, however, that only analytical work of a high order of accuracy would suffice to discriminate between the two possibilities even if the preparations were pure, and, in any case, salts of optically active isocitric acid seldom yield satisfactory analytical results. The specific rotation of a sample of the acid dissolved in water was $+9.4^\circ$; when the solution was concentrated to about one-tenth its volume, the specific rotation was found to be $+5.9^\circ$. The solution was then evaporated to a sirup and again dissolved in water; the specific rotation was now -6.1° . This solution, after a few days at room temperature, became nearly optically inactive, but after it had been refluxed for 10 hours the specific rotation had risen again to $+7.9^\circ$. Another specimen of the acid was evaporated *in vacuo* to a sirup, dissolved in acetone, again concentrated, and the residue was dried to constant weight at 110° . The product, dissolved in acetone, had a specific rotation of -39.5° . Aberson records these curious observations without attempting to account for them. The behavior is, however, what might be expected of a γ -hydroxy acid that is readily converted in part into its lactone, and has been duplicated with preparations of isocitric acid (11). According to Bruce (12) the specific rotation of pure isocitric acid is $[\alpha]_{589}^{26} = +17.7^\circ$, while that of the lactone is -62° under the same conditions. The anhy-

drides of *l*-malic acid are much less strongly levorotatory than this, the extreme figure recorded by Walden (9) for a product obtained by heating malic acid to a high temperature being about -17° . There seems little doubt, therefore, that Aberson's crude material must have contained much isocitric acid.

Further evidence of this comes from a consideration of his data on a preparation purified by distillation of the methyl esters. From 20 gm. of crude esters, about 8 gm. distilled at or near 161° at 24 mm. pressure. This

TABLE I

Theoretical Composition of Isocitric Acid Derivatives and of Malic Acid Derivatives Compared with Data of Aberson (7)

Figures not otherwise designated are percentages.

	Theory for isocitric acid derivative	Theory for malic acid derivative	Found by Aberson
Silver salt	$C_6H_5O_7Ag_2$	$C_6H_5O_7Ag_2$	
C	14.05	13.80	13.8, 13.6, 13.4
H	0.98	1.16	1.1, 1.2, 1.2
Ag	63.11	62.02	62.1, 62.4, 63.2
Lead salt	$(C_6H_5O_7)_2Pb_2$	$C_6H_5O_7Pb$	
C	14.42	14.16	14.8
H	1.01	1.19	1.2
Pb	62.18	61.06	61.0
Dimethyl ester (high b.p. fraction)	Lactone $C_8H_{10}O_4$	Cyclic anhydride $C_8H_{10}O_4$	
C	47.52	46.15	46.2, 45.9, 46.4, 46.2, 46.1
H	4.98	4.64	5.0, 5.1, 5.0, 4.9, 4.8
M.p., $^\circ C$.	107	102 (9)	102
Free acid recovered from distilled methyl ester and dehydrated	Lactone $C_8H_{10}O_4$	Cyclic anhydride $C_8H_{10}O_4$	
C	41.40	41.40	41.1
H	3.47	3.47	3.8
Mol. wt.	174.1	232.1	196

was fairly convincingly identified as a substance of the composition of dimethyl malate; Aberson regarded it as crassulacean malic acid ester. A second fraction was then obtained which distilled at about 210° at the same pressure; this was crystalline and, after recrystallization from alcohol, melted at 102° . The dimethyl ester of isocitric lactone melts at about 107° . Aberson's analyses of this product are given in Table I. He interpreted them as evidence of the transformation of crassulacean malic acid into its cyclic anhydride. Walden later (9) prepared the methyl ester of an

anhydride of authentic malic acid and found it to melt at 102° , but when the behavior with respect to optical rotation of Aberson's material is taken into consideration, there seems little reason to doubt that his preparation was a moderately pure specimen of dimethyl isocitrate lactone. Aberson prepared the free acid from this ester and dehydrated it thoroughly; the analysis (Table I) throws no light on the matter, since the percentage composition of isocitric lactone is identical with that of malic anhydride, but his molecular weight determination agrees appreciably better with the assumption that the product was mainly an isocitric acid derivative. Furthermore his description of the slow crystallization of the sirup when preserved in a desiccator corresponds exactly with the behavior of isocitric lactone.

Aberson's further efforts to characterize this specimen of the free acid are not helpful in reaching a decision as to its identity. He prepared silver salts by several techniques, and one of these products agreed almost exactly in composition with the theory for the malic acid salt; other preparations, however, were much too low in silver content and may have represented partially dehydrated material or mixtures of the lower silver salts.

An examination of Franzen and Ostertag's data likewise lends color to the idea that they had moderately pure specimens of isocitric acid in hand. The ester fractions of high boiling point they describe may well have contained derivatives of ordinary *l*-malic acid; this is an experience that has been encountered in this laboratory (13), since it is difficult to provide against the formation of small amounts of what appear to be self-esters or partial anhydrides of malic acid during the usual process of esterification, and these products, if present, are later found in the fractions that distil in the temperature range in which the esters of citric acid (and also isocitric acid) come over. But in describing the preparation of the hydrazide from these fractions, Franzen and Ostertag state that, after the reagent was added to the alcoholic solution of the ester, no evidence of reaction was apparent for a short period, when, quite suddenly, the solution became turbid and in a few moments was filled with a crystalline product. This behavior was new to them in spite of their extensive experience with the hydrazides of all the common plant acids. It is invariably observed with ester fractions that are rich in isocitric acid, but its significance escaped the German investigators. The isolation of a product that melted at $178-179^{\circ}$, *i.e.* the melting point of malic dihydrazide, apparently quieted any suspicion they may have entertained of the identity of their product. However, their statement that the decomposition point was not depressed by the admixture of malic dihydrazide remains unexplained.

It should be pointed out that their yield of 0.97 gm. of presumed malic acid dihydrazide from 1.0 gm. of ester might well have struck them as being

peculiar. The theoretical yield would be 0.85 gm. if the material had indeed been malic ester and they thus isolated nearly 114 per cent of the expected amount. On the other hand, if their ester preparation were mainly the diethyl ester of isocitric lactone, the theoretical yield of trihydrazide would be about 1.02 gm. and they obtained 95.3 per cent of this quantity.

DISCUSSION

The identification of the so called crassulacean malic acid as isocitric acid probably raises more problems than it solves. The observation contributes little that is immediately apparent to an explanation of the unusual organic acid metabolism of the succulents, a problem that has attracted more attention over a far longer period of time than have similar problems for any other botanical group, and which is still unsolved. The diurnal variation in the organic acid acidity of *Bryophyllum* leaves appears, from the preliminary data that have been accumulated, to be in fact largely a matter of rapid changes in the quantities of *l*-malic acid present. The extent to which isocitric acid may share in these changes remains to be determined.

Accordingly the problem is merely redefined. It will be necessary, ultimately, to secure evidence upon the nature of the equilibria between the several organic acids in these tissues, and to obtain information concerning the enzyme systems which control the reactions that take place, a program that will doubtless require years of study in many laboratories. The present paper is written in the hope that attention will be directed to the fundamental nature of this problem, since there is little doubt that the explanation, once obtained, will have a direct bearing upon the problem of the respiration of living cells.

SUMMARY

An examination of the literature of the so called crassulacean malic acid has shown that there is every likelihood that early workers with this substance had more or less impure specimens of isocitric acid in hand. The composition of the salts and other derivatives of isocitric acid is so closely similar to that of the similar compounds of malic acid that confusion could scarcely be avoided unless strictly homogeneous preparations were obtained and analyzed with the utmost accuracy. This does not seem to have been accomplished. Furthermore, although isocitric acid was known as an optically inactive product of synthesis throughout the period during which discussions of crassulacean malic acid occasionally appear in the literature, the optically active isomer had not been encountered and there was accordingly no information available upon the strikingly characteristic behavior of this substance.

BIBLIOGRAPHY

1. Franzen, H., and Keyssner, E., *Biochem. Z.*, **135**, 183 (1923).
2. Mayer, A., *Landw. Versuch.-Stat.*, **18**, 410 (1875).
3. Mayer, A., *Landw. Versuch.-Stat.*, **21**, 277 (1878).
4. Mayer, A., *Landw. Versuch.-Stat.*, **34**, 127 (1887).
5. Bennet-Clark, T. A., *New Phytologist*, **32**, 39, 128, 197 (1933).
6. Schmidt, E., *Arch. Pharm.*, **24**, 535 (1886).
7. Aberson, J. H., *Ber. chem. Ges.*, **31**, 1432 (1898).
8. Werner, A., *Lehrbuch der Stereochemie*, Jena, 114 (1904).
9. Walden, P., *Ber. chem. Ges.*, **32**, 2706 (1899).
10. Franzen, H., and Ostertag, R., *Z. physiol. Chem.*, **122**, 263 (1922).
11. Pucher, G. W., *J. Biol. Chem.*, **145**, 511 (1942).
12. Bruce, W. F., *J. Am. Chem. Soc.*, **57**, 1725 (1935).
13. Vickery, H. B., and Pucher, G. W., *Connecticut Agric. Exp. Stat., Bull.* **352** (1933).

AN ETHER EXTRACTION METHOD FOR THE DETERMINATION OF URINE PHENOLS

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Experiments previously reported from this laboratory (1) have shown that less than 1 per cent of the "total diazo" value of the blood is actually made up of phenols, the reactive substances being almost exclusively ether-insoluble and presumably nitrogenous in nature¹ (2). In the present work, an attempt was made to develop more specific methods for the determination of the various groups of urinary phenols. This objective was attained by continuous ether extraction of urine at different pH levels. In this manner the more truly phenolic bodies are separated from the ether-insoluble but diazo-reactive substances in the urine. In addition, these phenols and aromatic hydroxy acids are obtained in separate fractions available for analysis with diazotized *p*-nitroaniline.

Apparatus and Reagents—

Interchangeable glass joints are necessary, since corks or rubber connections persistently yield phenolic or diazo chromogenic material.

The extraction apparatus consists of four parts. (1) A 500 mm. No. 2800 West type of condenser with outer standard taper² 24/40 joint at the top, and inner 24/40 joint at the bottom; a No. 7580 solid glass stopper (standard taper 24) rests *loosely* in the top of the condenser.³ (2) The extraction tube,⁴ which consists of a 30 mm., outside diameter, tube narrowed at the upper end and sealed to the outer part of a 24/40 interchangeable joint. Into the lower end of the 30 mm. tube is sealed, coaxially, an 11 mm., outside diameter, vapor flow and liquid return tube. This tube extends upward about 125 mm. and downward 60 mm. to the 14/35 inner part to which it is sealed. The length of the body of the large extraction tube is

¹ Experiments are in progress in this laboratory on an improved method for the determination of blood phenols, on the basis of information derived from the present work.

² Pyrex, standard taper.

³ A Liebig type of condenser may be used, but it does not seem to be as efficient, especially in hot weather. Condensers, flasks, interchangeable joints, etc., are carried in stock by the Corning Glass Works, Corning, New York (Catalogue LP21, 1941). The entire extraction apparatus can be obtained on special order from E. J. Callahan and Company, 14 West Barre Street, Baltimore.

⁴ The special coaxial extraction tube was designed by Dr. H. B. Wylie of this department and made by E. J. Callahan and Company.

about 230 mm. and has a total capacity of 48 to 50 cc. A diagram (but no description) has previously been published (1). (3) A piece of 6 mm., outside diameter, tubing shaped like a small funnel at the top and drawn to a small opening at the bottom. (4) The receiver is an 18 × 150 mm. Pyrex culture tube No. 9820, to the open end of which is sealed the outer part of a 14/35 joint. It is graduated at 3 cc. intervals. A smaller receiver tube made from a 13 × 100 mm. Pyrex culture tube with a 14/35 joint and graduated at 3 cc. is also necessary.³

Ether. The ether should be distilled from glass almost daily, since it rapidly develops chromogenic substances upon exposure, which react with the diazo reagents. Distilled water containing a few drops of 0.1 N NaOH or 10 N H₂SO₄ can be extracted continuously with ether for several hours without the production of such chromogenic material, if the top of the condenser is kept closed with a solid glass stopper.

Diazotized color reagent. 25 cc. of the *p*-nitroaniline solution (1.5 gm. of the crystalline material in 500 cc. of water containing 40 cc. of concentrated HCl) are added to 1.5 cc. of a 5 per cent sodium nitrite solution. This reagent will remain clear and reactive for several days if kept in the cold.

Standard solutions of phenols and aromatic hydroxy acids for color comparison. A stock phenol solution is prepared by dissolving 1 gm. in 95 per cent alcohol and diluting to 1000 cc. Various standards are then prepared containing 0.001, 0.005, 0.01, 0.05, and 0.1 mg. of phenol per cc., respectively, by proper dilution with alcohol of accurately measured amount of the stock solution.

A stock solution of *p*-cresol in alcohol is also prepared containing 1 mg. per cc. A stock phenol-*p*-cresol solution is prepared by diluting 10 cc. of the alcoholic phenol and 40 cc. of the alcoholic *p*-cresol to 100 cc. with alcohol. Each cc. thus contains 0.5 mg. of phenol-*p*-cresol. Appropriate standards are prepared from this solution.

A stock solution of *p*-hydroxybenzoic acid in alcohol is prepared containing 1 mg. per cc. from which standards are prepared ranging from 0.001 to 0.1 mg. per cc. A solution containing 1 mg. of *p*-hydroxyphenylacetic acid per cc. is also made up in alcohol. Dilute 16.67 cc. of the *p*-hydroxybenzoic acid and 50 cc. of the *p*-hydroxyphenylacetic acid stock solutions to 100 cc. with alcohol. Each cc. contains 0.667 mg. of the mixed acids. Prepare appropriate standards by dilution with alcohol.

Reaction of Diazotized p-Nitroaniline with Various Phenolic Bodies

Diazotized *p*-nitroaniline, the color reagent, reacts with a variety of phenols and aromatic hydroxy acids. Therefore a preliminary study was made of the shade and amount of color given by fractional milliequivalen

quantities of phenol, *p*-cresol, catechol, resorcinol, phloroglucinol, *p*-hydroxybenzoic acid, *p*-hydroxyphenylacetic acid, and *o*-coumaric acid in which phenol was used as a standard for comparison. The various tests were performed under experimental conditions similar to those described later in this paper for the determination of the phenolic bodies in urine.

It was found that no consistent relationship exists between the molecular structure of the compounds studied and the amount of color given by them with the diazo reagents. Although equivalent quantities of phenol and *p*-hydroxybenzoic acid gave the same amount of orange-red color, most of the compounds studied gave solutions of varying shades of brown, colors which could not be matched readily with the phenol standard. *p*-Cresol, the most abundant of the volatile urinary phenols, yielded a violet-brown color of an intensity about one-third that given by phenol. In addition, like experiments carried out in alcohol, water, alcohol-water, and alcohol-water-ether media gave different quantities of color. Obviously, the actual numerical value reported for the phenolic content of urine will depend on the composition of the standard used for comparison.

Preliminary Experiments with Phenolic Compounds in Aqueous Solution

Medium for Diazo Reaction—Previously it was suggested that the incomplete recovery of phenol added to blood was due in part to loss during volatilization of the ether (1). Further experiments showed that when 1 cc. portions of various solutions are shaken with 5 cc. of ether and then subjected to evaporation, 33 to 50 per cent of the phenol and *p*-cresol and 5 to 11 per cent of the catechol and resorcinol are carried away by the ether vapors. However, *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, and β -resorcylic acids suffered no loss. Various expedients including extraction of the phenolic bodies from the ether with aqueous alkaline solutions proved cumbersome and unsatisfactory. Finally it was found that these compounds could be determined directly in the ether extract by the addition of alcohol, water, and the various diazo reagents. This innovation, the addition of alcohol to produce a homogeneous solution, has simplified the problem immensely.

Effect of pH on Extraction of Phenolic Bodies—Aqueous stock solutions of the various compounds undergoing this examination were prepared to contain 1 mg. per cc. Aliquot quantities were then transferred to 100 cc. flasks, water was added, and the pH⁵ of the various solutions adjusted with 0.1 \times NaOH or \times H₂SO₄ to values ranging from 1 to 12, and the contents then made up to 100 cc. 40 cc. of each solution, containing amounts of

⁵ All pH values reported in this paper were obtained with a Beckman glass electrode pH meter, model G, manufactured by the National Technical Laboratories, Pasadena, California.

material varying from 0.005 to 1 mg., were transferred accurately to extractors and extracted continuously for 2 hours. The resultant ether extracts were analyzed according to the procedures outlined later in this paper for urinary phenols.

It was found that phenol and *p*-cresol in various concentrations are recovered quantitatively not only from acid solution, but from mildly alkaline solutions up to pH 10 to 10.5 as well. Catchol, resorcinol, and phloroglucinol are also completely extracted from acidified solutions, but in slightly alkaline media recoveries are frequently incomplete in the time allowed for the extractions. The more strongly acidic *p*-hydroxybenzoic, *p*-hydroxyphenylacetic, gallic, *o*-coumaric, and β -resorcylic acids are not extracted from faintly alkaline solutions and even in slightly acid media recovery is poor. However, at a pH of 3 or lower, these acids are completely extracted by the ether.

The following experiments show that phenols and aromatic hydroxy acids can be quantitatively separated by ether extraction at selective pH. Accurate quantities of the aqueous stock phenols and aromatic hydroxy acid solutions were transferred to volumetric flasks, water was added, and the solutions adjusted to pH 10 (± 0.2) with a few drops of 0.1 N NaOH and diluted to 100 cc. 40 cc. portions, containing the amounts of reactive bodies as given in Table I, were extracted continuously for 2 hours and the contents of the ether extracts analyzed by procedures outlined later for the urine phenols. 1 cc. portions of 10 N H_2SO_4 were added to each extractor and the residues (now at pH 1) were subjected to extraction and analysis according to the procedures outlined later for the aromatic hydroxy acids in urine. The data in Table I show that such mixtures of phenol and *p*-cresol and the aromatic hydroxy acids can be readily separated and their respective concentrations quantitatively determined in the ether extracts. The diphenols were not completely extracted at pH 10 under these conditions. However, their concentration in urine is not large.

Procedures for Analysis of Phenolic Bodies in Urine

Procedure for Free Phenols—20 cc. of urine are transferred to a 100 cc. volumetric flask, water is added, and the solution adjusted to pH 10 (± 0.2) with 0.1 N NaOH and then diluted to the mark with water. 40 cc. are transferred to the coaxial extraction tube and freshly distilled ether (about 9 cc.) is added until the ether layer just overflows the inner return tube. A few particles of pulverized porcelain (to prevent bumping) and about 3 cc. of ether are added to the small receiver tube, which is then attached to the extraction tube and immersed in water maintained at 70–75°. The solid glass stopper is placed *loosely* in the top of the condenser. Small bubbles of ether rapidly pass upward through the diluted urine. A rapid,

continuous extraction is maintained for 2 hours. The water bath is removed and the receiver tube allowed to cool for a few minutes. The apparatus is shaken gently until sufficient ether overflows the inner return tube to bring the contents of the receiver up to the 3 cc. mark. The ether extract is now diluted with 5 cc. of 95 per cent alcohol. 5 cc. of an alcoholic standard containing 0.001 mg. of phenol and 0.004 mg. of *p*-cresol (0.005

TABLE I

Separation and Analysis of Known Mixtures of Phenols and Aromatic Hydroxy Acids

Phenolic compound extracted at pH 10			Aromatic hydroxy acid extracted at pH 1		
Name	Amount	Recovery	Name	Amount	Recovery
	mg. per 40 cc.	per cent		mg. per 40 cc.	per cent
Phenol	0.1	98	<i>p</i> -Hydroxybenzoic acid	0.4	97
"	0.4	94	" "	0.4	103
"	0.1	102	" "	0.1	103
"	0.4	96	" "	0.1	107
"	0.1	105	<i>p</i> -Hydroxyphenylacetic acid	0.4	103
"	0.4	104	" "	0.4	97
"	0.1	96	" "	0.1	109
"	0.4	102	" "	0.1	119
<i>p</i> -Cresol	0.1	97	<i>p</i> -Hydroxybenzoic acid	0.4	105
"	0.4	97	" "	0.4	103
"	0.1	103	" "	0.1	103
"	0.4	98	" "	0.1	104
"	0.1	98	<i>p</i> -Hydroxyphenylacetic acid	0.4	95
"	0.4	100	" "	0.4	98
"	0.1	103	" "	0.1	93
"	0.4	104	" "	0.1	94
Resorcinol	0.1	108	<i>p</i> -Hydroxybenzoic acid	0.4	104
"	0.4	93	" "	0.4	104
"	0.1	104	" "	0.1	100
"	0.4	95	" "	0.1	112
"	0.1	96	<i>p</i> -Hydroxyphenylacetic acid	0.4	118
"	0.4	92	" "	0.4	106
"	0.1	70	" "	0.1	147
"	0.4	69	" "	0.1	127

mg. of the mixture) and 5 cc. of another alcoholic standard containing 0.003 mg. of phenol are pipetted into separate test-tubes. Now add 2 cc. of distilled water, 1 cc. of diazotized *p*-nitroaniline, and 3 cc. of 5 per cent sodium carbonate to each tube. All solutions and apparatus must be free from any contamination. A straw-yellow color, not much greater than that given by a blank control, appears at once. The concentration of

these standards is usually sufficient for normal urine. Since the reaction shows poor proportionality at this low concentration, due mainly to the fact that the reagents themselves yield appreciable amounts of color, standard and unknown should have approximately the same concentration. When the solution stands longer than 10 minutes, color intensification occurs, owing to the continued reaction between the reagents. In more concentrated solutions such as occur with the other urinary fractions, this color intensification is hardly noticeable.

Procedure for Free Aromatic Hydroxy Acids—The reaction of the residue in the extractor, which has remained around pH 10 during the extraction, is reduced to about pH 1 by the addition of 1 cc. of 10 N H_2SO_4 . To the large graduated receiver are added a few particles of pulverized porcelain and about 9 cc. of ether. Extraction is carried on for 2 hours. The extract is adjusted to the 9 cc. mark with ether and then diluted with 15 cc. of alcohol. 8 cc. of this mixture are removed for analysis. 5 cc. portions of appropriate alcoholic standards, generally containing 0.1 mg. of phenol-*p*-eresol mixture, 0.133 mg. of *p*-hydroxybenzoic-*p*-hydroxyphenylacetic acids, and 0.1 mg. of *p*-hydroxybenzoic acid, respectively, are transferred to separate test-tubes and 3 cc. of ether are added to each standard. All three standards need not necessarily be prepared. After addition of 2 cc. of water, 1 cc. of diazotized *p*-nitroaniline, and 3 cc. of 5 per cent sodium carbonate, maximal color development occurs at once and remains unchanged for nearly 30 minutes. The *p*-hydroxybenzoic acid standard yields an orange-red color and *p*-hydroxyphenylacetic acid a violet-brown color, but a 1:3 mixture of these two acids gives a reddish brown color similar to that given by the ether extract. Phenol-*p*-eresol also gives a good match. The reaction is complicated by the fact that the individual compounds produce different shades and amounts of color with the diazo reagents. Hence the numerical value reported for the urinary aromatic hydroxy acids, and other groups or fractions as well, will be an arbitrary figure, depending upon the standard used for comparison. The standard is placed at 20 mm. in the colorimeter. The values for each fraction are obtained by the usual colorimetry calculations.

Accurate results are obtained only if the standard and unknown contain approximately like amounts of color. Frequently the ether extract may contain larger amounts of free hydroxy acids. In such cases a measured amount of the ether-alcohol extract is diluted to 8 cc. with a 3:5 ether-alcohol mixture; so that the required standard will not exceed 0.1 mg. of phenol-*p*-eresol mixture, for example. Otherwise values which are too high may be obtained. This point was observed particularly in experiments in which added *p*-hydroxybenzoic acid was being recovered from urine.

The ether extracts a maximal quantity of free acids when the diluted

urine has a pH of 1. Progressively lower yields are obtained as the pH is raised. Second and even third extractions may contain a small but measurable amount of reactive acids. Although the temperature of the urine rises to 30–35° during the extraction, it was found that appreciable deconjugation does not occur until a temperature well above 45° is attained and does not become complete until the temperature approaches 100°. This information was obtained by allowing preparations of diluted urine at pH 1 to stand for 2 hours at temperatures ranging from 20–100° before extraction with ether. Apparently acidified urine yields a small amount of slowly extractable but reactive material. This is not observed when hydrolyzed urine is extracted.

Procedure for Conjugated Phenols—The extracted residue is decanted into a 100 cc. flask (No. 4320)² which has a 24/40 joint. The extraction tube is rinsed with two 10 cc. portions of water which are also poured into this flask. Several glass beads are added and the ether volatilized from a hot water bath. The flask is attached to a condenser and the contents gently boiled for 1 hour. After cooling, 11.2 cc. of N NaOH are added. Generally this amount of alkali will bring the hydrolysate up to approximately pH 10. The solution is diluted with water to 80 cc. It is well to check the pH. 40 cc. are extracted rapidly for 2 hours with ether. The extract is made up to 9 cc., diluted with 15 cc. of alcohol, and 8 cc. are removed for analysis. 5 cc. portions of alcoholic standards containing 0.1 mg. of the phenol-*p*-cresol mixture and 0.05 mg. of phenol, respectively, are transferred to separate test-tubes containing 3 cc. of ether. After addition of the diazo reagents a deep reddish brown color immediately develops and matches well that given by the phenol-*p*-cresol standard, but does not match the orange-red color of the phenol nor the brown color of the *p*-cresol standards. 97 to 100 per cent of the deconjugated phenols is removed by the 2 hour extraction. Hence a second extraction is not necessary.

Procedure for Conjugated Aromatic Hydroxy Acids—1 cc. of 10 N H_2SO_4 is added to the residue, extraction continued for 3 hours with 9 cc. of ether, and the extract then analyzed according to the procedure for the free aromatic acids. A second extraction is unnecessary. If the residue is removed, hydrolyzed for an additional hour, and again extracted, appreciable quantities of reactive bodies are not obtained. Hence the initial hydrolysis produces complete deconjugation.

Procedure for Total Phenols and Aromatic Hydroxy Acids Together—20 cc. of urine and 22 cc. of N H_2SO_4 are transferred to a volumetric flask and diluted to 100 cc. with water. The pH will be close to 1. The contents are poured into a 200 cc. flask (No. 4320).³ A few glass beads are added and the contents refluxed gently for 1 hour. A slight decrease in acidity occurs owing to conversion of urea into ammonia.

During hydrolysis, the diluted urine acquires a straw color, which is partly soluble in ether. Occasionally an additional ether-soluble pink pigment is formed which seems to react with the diazo reagents. It has been suggested that resorcinol may give a red substance with indoxyl under these conditions (3). These pigments do not offer serious interference, however.

10 cc. of the cooled hydrolysate, 1 cc. of 10 N H_2SO_4 , and 30 cc. of water are extracted for 3 hours. The extract is made up to the 9 cc. mark and diluted with 15 cc. of alcohol. After addition of the usual reagents to 8 cc. of this solution, a deep reddish brown color develops which matches well that given by the phenol-*p*-cresol and by the *p*-hydroxybenzoic-*p*-hydroxyphenylacetic acid standards, but not those prepared from the individual compounds.

Maximum yields of phenolic bodies are obtained following hydrolysis for 1 hour at pH 0.5 to 1. However, at pH 2 and higher deconjugation is incomplete. 97 to 99 per cent of the total phenols and aromatic hydroxy acids in the hydrolysate is removed by 3 hours of rapid extraction with ether.

Procedure for Separation and Determination of Total Phenols—50 cc. of hydrolysate are pipetted into a volumetric flask containing about 35 cc. of water. This solution is then adjusted to pH 10 (± 0.2) by the addition of 11.7 cc. of N NaOH. It is well to check this value before final dilution to 100 cc. 40 cc. of the alkalinized hydrolysate are extracted for 2 to 3 hours in the usual manner. The extract is made up to 9 cc., diluted with 15 cc. of alcohol, mixed, and 8 cc. removed for analysis. The addition of 2 cc. of water and the diazo reagents produces a deep, reddish brown color which matches perfectly that given by a phenol-*p*-cresol standard having the same concentration of reactive material. The orange-red color yielded by a phenol standard makes a poor match, however.

Procedure for Determination of Total Aromatic Hydroxy Acids—1 cc. of 10 N H_2SO_4 is added to the residue and extraction resumed for 3 hours. The extract is made up to 9 cc., 15 cc. of alcohol are added, and 8 cc. removed for analysis. The deep reddish brown color which develops gives a perfect match with the phenol-*p*-cresol or the *p*-hydroxybenzoic-*p*-hydroxyphenylacetic acid standards, but not with the individual standards.

Effect of Standing at Room Temperature and at Ice Box Temperature on Urinary Phenolic Bodies

A fresh 24 hour specimen of urine (1000 cc.) was subjected to complete analysis according to the various procedures outlined above. Then part of the urine was allowed to stand at room temperature (15–36°) and the remainder in the refrigerator in glass-stoppered bottles. Samples were removed from each bottle after 2, 6, 15, and 27 days for analysis. In some

cases phenol determinations were also made by the Folin-Denis method (4). Table II clearly shows that, even after 27 days, no appreciable change in the concentration of the various phenolic and aromatic hydroxy acid fractions occurs in the refrigerated urine. Likewise no change takes place for at least 2 days in the urine kept at room temperature. The most noticeable change which occurred in that urine, thereafter, was a progressively enormous increase in the free phenols, the concentration rising from 0.7 to 82 mg. of phenol-*p*-cresol at the end of 27 days. This increase was due mainly to deconjugation and possibly some tyrosine decomposition, since a moderate increase in total phenols also occurred. No change in the total aromatic hydroxy acids was observed, although some deconjugation is evident. These observations are quite in contrast to reports (5, 6) that the aromatic hydroxy acids are converted into free *p*-cresol and phenol in urine standing 5 to 6 days.

Recovery of Phenol and p-Hydroxybenzoic Acid Added to Urine—The free phenol and aromatic hydroxy acid content of a specimen was determined in the usual manner. Measured amounts of aqueous phenol and *p*-hydroxybenzoic acid stock solutions were added to 20 cc. portions of the same urine. The solutions were made up to pH 10 and diluted to 100 cc. 40 cc. portions were then analyzed as above.

Table III shows that good phenol recoveries were effected regardless of the amount added to the urine. *p*-Hydroxybenzoic acid recoveries were fair, but tended to be high. This irregularity may be due to the fact that the standard had an orange-red color, whereas the urine preparation containing a mixture of compounds was orange-brown. In the more concentrated urines, an aliquot of the extract (to which alcohol was added) had to be diluted with the ether-alcohol mixture before analysis in order to secure accurate results.

Analysis of Ten 24 Hour Specimens of Normal Urine—The maximal, minimal, and average values obtained for each of the seven urinary groups are given in Table IV. It is at once apparent that the free phenols are negligible in amount (0.2 to 0.4 mg. of phenol per 24 hour specimen). The conjugated phenols average 30.7 mg. of phenol or 63.9 mg. of phenol-*p*-cresol as compared to 30.6 and 65.7 mg., respectively, for the total phenols when determined together on the hydrolyzed urine. Of the total aromatic hydroxy acids, approximately two-thirds occurs free and one-third conjugated. Thus the free aromatic hydroxy acids for the ten 24 hour specimens average 75 mg. and the conjugated acids 41.5 mg., making a total of 116.5 mg. as *p*-hydroxybenzoic-*p*-hydroxyphenylacetic acids, as compared to 107.7 mg. when determined together. These relations check with the fact that the phenols are toxic, whereas the aromatic hydroxy acids are relatively non-toxic. The average value for the total phenolic bodies is

161 mg. of phenol-*p*-cresol or 78.4 mg. of phenol as compared to 356 mg. of phenol obtained with the Folin-Denis (4) method on unextracted urines. The average for the sum of the free phenols, free aromatic hydroxy acids, conjugated phenols, and conjugated aromatic hydroxy acids checks well with the average value obtained for the total bodies when determined together.

TABLE II

Effect of Standing at Room Temperature and at Ice Box Temperature on the Various Urinary Phenols

All determinations by the ether extraction methods are recorded as mg. of phenol-*p*-cresol per 1000 cc. of urine. All determinations by the Folin-Denis method are recorded as mg. of phenol per 1000 cc. of urine.

Days	Free phenols		Free aromatic hydroxy acids		Conjugated phenols		Conjugated aromatic hydroxy acids		Total phenols		Total aromatic hydroxy acids		Free "phenols" by Folin-Denis method		Total "phenols" by Folin-Denis method	
	Room	Ice box	Room	Ice box	Room	Ice box	Room	Ice box	Room	Ice box	Room	Ice box	Room	Ice box	Room	Ice box
0	0.7	0.7	48	48	50	50	19	19	51	51	58	58	235	235	367	367
2	0.8	0.8	48	48	51	53	20	21	54	52	54	53				
6	8.0	0.8	53	47					58	54	54	56	241	250	350	356
15	26.0	0.6	46	44					63	54	55	56	258	243	364	330
27	82.0	0.6	64	49	11	55	1	14	72	54	54	60	333	228	320	208

TABLE III

*Recovery of Phenol and *p*-Hydroxybenzoic Acid Added to Urine*

The values (except per cent recovery) are expressed as mg. per 1000 cc. of urine.

Experiment No.	Free phenol in urine	Phenol added	Phenol found	Phenol recovered	Per cent recovery	Free <i>p</i> -hydroxybenzoic acid in urine	<i>p</i> -Hydroxybenzoic acid added	<i>p</i> -Hydroxybenzoic acid found	<i>p</i> -Hydroxybenzoic acid recovered	Per cent recovery
1	0.5	150	145.0	144.5	96.3	95.0	150	267	172	114.7
2	0.5	25	24.5	24.0	96.0	55.0	50	113	58	116.0
3	0.5	5	5.4	4.9	98.0	50.0	25	77	27	108.0
4	0.3	2	2.0	1.7	85.0	50.0	25	76	26	104.0

It is obvious that the actual values reported for any group depend upon the method and the composition of the standard used for comparison. Thus, the values (as phenol) for the total diazo-reactive bodies, obtained by the Folin-Denis method directly on the urines, are approximately double the values obtained by the present ether extraction method for total phenolic bodies, when reported as phenol-*p*-cresol, and about 4 times these values when reported as phenol.

TABLE IV
Complete Analysis of Ten 24 Hour Specimens of Normal Human Urine (Mg. per 24 Hour Specimen)

Urine No	Free phenols		Free aromatic hydroxy acids				Conjugated phenols			Conjugated aromatic hydroxy acids			Total bodies		Total phenols		Total aromatic hydroxy acids				Total aromatic hydroxy acids (a) + (c) + (d)	Total bodies (e) + (f) + (g) + (h) + (i)	Folin-Denis method	
	(a)	(b)	Phenol-p-cresol		(c)	(d)	Phenol		(e)	(f)	Phenol-p-cresol		(g)	(h)	(i)	(j)	(k)	(l)	(m)	(n)	(o)	(p)	(q)	(r)
			(a)	(b)			(c)	(d)			(e)	(f)												
1	0.5	0.2	06	83	60	00	30	31	39	21	175	88	70	37	90	110	71	69	97	166	5	250	384	
2	0.6	0.3	81	108	62	38	10	38	17	29	156	75	10	15	120	150	101	38	119	157	6	318	112	
3	0.5	0.3	75	102	60	31	38	33	11	27	209	100	87	10	110	130	70	81	108	102	5	170	220	
4	0.5	0.2	47	59	40	37	16	16	18	11	107	70	10	16	58	70	38	37	53	100	5	275	387	
5	0.8	0.1	50	68	48	05	30	22	29	15	157	70	60	31	76	97	18	65	81	116	8	360	460	
6	0.0	0.3	12	55	38	60	37	31	48	19	132	86	59	25	01	81	55	60	73	133	6	250	360	
7	0.8	0.3	61	83	55	82	15	57	08	10	108	91	90	11	115	110	100	100	82	121	203	8	370	470
8	0.8	0.1	50	01	45	83	10	50	60	39	20	177	89	85	10	101	122	88	83	100	183	8	370	470
9	0.8	0.1	37	11	29	18	22	26	33	20	119	00	17	21	52	61	11	18	03	111	8	370	470	
10	0.5	0.2	70	87	01	71	33	31	12	25	180	80	73	10	95	110	70	73	101	177	5	370	470	
Maximum	0.8	0.1	81	108	69	81	15	57	68	40	209	100	90	11	120	150	101	81	121	203	8	318	412	
Minimum	0.5	0.2	37	11	29	37	10	16	18	11	107	19	10	15	62	01	38	37	53	100	5	170	220	
Average	0.61	0.3	59	75	51	63	930	733	8	11	101	78	165.7	30.6	88	107	71	71	91	157	1	255	356	

SUMMARY

An analytical method for urine phenols is described in which these bodies are extracted with ether at selective pH and then determined directly in the various ether extracts with diazotized *p*-nitroaniline. The extractions are carried out in an all-glass apparatus which utilizes a newly designed coaxial extraction tube. The ether-soluble phenolic bodies in the urine are separated into seven groups: free, conjugated, and total phenols; free, conjugated, and total aromatic hydroxy acids; and finally, total phenolic bodies. An important innovation, dilution with alcohol, made possible the determination of these groups of compounds directly in the ether extract. Recovery of phenols and aromatic hydroxy acids added to aqueous solutions and to urine was effected in a satisfactory manner.

It was found that various phenolic compounds give different shades and quantities of color with the diazo reagents; hence the actual numerical value for the various groups of urinary phenols obtained by the reported procedures depends upon the standards used for comparison.

Appreciable changes in the concentration of the various groups of urinary phenolic bodies are not observed in urine kept in the cold, even after 27 days. After 2 days at room temperature, however, the concentration in free phenols increased greatly. Conversion of aromatic hydroxy acids into *p*-cresol and phenol under these conditions, as stated in the literature, was not observed.

BIBLIOGRAPHY

1. Schmidt, E. G., Schmulovitz, M. J., Szczpinski, A., and Wylie, H. B., *J. Biol. Chem.*, **120**, 705 (1937).
2. Deichmann, W., and Schafer, L. J., *Am. J. Clin. Path.*, **12**, 129 (1942).
3. Rose, A. R., and Exton, W. G., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **109**, p. lxxvi (1935).
4. Folin, O., and Denis, W., *J. Biol. Chem.*, **22**, 305 (1915).
5. Tisdall, F. F., *J. Biol. Chem.*, **44**, 409 (1920).
6. Baumann, E., *Ber. chem. Ges.*, **12**, 1452 (1879).

CONCERNING THE REVERSIBLE INACTIVATION OF PROLAN

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Bowman (1) has reported that the activity of prolan, which has been decreased by boiling in aqueous solution, can be restored by exposure of the heat-treated material to hydroquinone at pH 8.5. The assumption is made that the inactivation by heat is the result of oxidation. The large percentage of recovery (between 33 and 50 per cent) of active material after boiling half an hour is contrary to the findings of other experimenters, who report considerably less than 10 per cent recovery and as low as a fraction of a per cent (2-4). The chemical picture of a reversible oxidation is an extremely interesting one; we have attempted without success to confirm it.

EXPERIMENTAL

A relatively crude prolan to conform with that used by Bowman was selected. The absence of estrogenic substances was assured by a negative uterine weight response in five castrated rats dosed with 4 times the maximum amount of material administered in any of the assays. The castrated rats were of the same age as the rats used for assay of prolan and the material was administered in exactly the same manner that was used for the latter assay.

The results of the experiments are shown in Table I.

Uterine weight increase served as the objective measure for assay of prolan. Female rats of the Evans strain, 22 days old, received three injections on successive days. The uteri were weighed 76 hours after the initial dose. Nine to eleven rats were used per dosage level in each assay. The dosage curve for 0, 0.01, 0.02, and 0.03 mg. per rat is given in Experiment I and served as the standard assay curve. In all crucial experiments the assay comparison is made between litter mates.

Since precipitation of the material treated with hydroquinone entails the assumption that chemical treatment has not changed the solubility properties, two experiments (Nos. IV and V) are included in which the treated material is diluted after the reaction time and given immediately. This procedure was controlled by adding hydroquinone to prolan just prior to dosage (Experiment II). Finally it was necessary to ascertain what effect

hydroquinone had upon prolan which had not been subjected to heat treatment. The heat inactivation of prolan was performed at 99°, pH 6.0, in isotonic phosphate-NaCl solution. The hydroquinone was employed in 1 per cent solution for 30 minutes at pH 8.5.

TABLE I

Treatment of Prolan with Hydroquinone before and after Heat Treatment

Ex- peri- ment No.	Treatment of prolan	Dose of prolan per rat	Uterine weight, mean and standard deviation of mean	Re- covery	Limit of per cent de- viation 19 of 20 times
		mg.	mg.	per cent	
I	Assay of untreated material	0	16 ± 1.0		
		0.01	26 ± 3.5		
		0.02	63 ± 8.4		
		0.03	99 ± 5.7		
II	Untreated prolan, hydroquinone before ad- ministration	0.03	93 ± 8.0	93	±20
	Hydroquinone 30 min.	0.03	36 ± 5.0	45	±11
III	10 min. at 99°, acetone pptn.	0.4	45 ± 7.5	4	±1
	10 " " 99°, hydroquinone 30 min.; ace- tone pptn.	0.4	29 ± 5.2	3	±1
IV	10 min. at 99°	0.3	34 ± 4.2	4	±1
	10 " " 99°	0.6	94 ± 6.5	5	±1
	10 " " 99°, hydroquinone 30 min.; dilu- tion, immediate administration	0.3	21 ± 2.1	<3	
V	2 min. at 99°	0.2	24 ± 2.5	5	±1
	2 " " 99°, hydroquinone 30 min.; dilu- tion, immediate administration	0.2	18 ± 0.8	<5	

Results

Experiments III and IV indicate that prolan heated in isotonic aqueous solution, pH 6.0, for 10 minutes loses 96 per cent of its activity. The three assay determinations agree within 1 per cent, this value being equivalent to twice the standard deviation of the mean. Reduction of the time of heating to 2 minutes still produced 95 per cent inactivation (Experiment V). Exposure of the heat-treated material to hydroquinone produced a lesser response to uterine stimulation in all cases (Experiments III, IV, V). The difference in response is significant in Experiments IV and V. Unheated prolan exposed to 1 per cent hydroquinone solution for 30 minutes lost approximately half of its activity (Experiment II). No loss of activity was detected when hydroquinone was added to prolan just prior to administration to the assay animals.

SUMMARY

In agreement with the findings of a number of workers, it was found that prolan lost more than 90 per cent of its biological activity on heat treatment at 99° in aqueous solution in as short a period as 2 minutes.

The biological activity of both intact and heat-treated prolan was decreased by treatment with hydroquinone at pH 8.5 for 30 minutes.

The reversible inactivation of prolan has not been confirmed.

BIBLIOGRAPHY

1. Bowman, D. E., *J. Biol. Chem.*, **137**, 293 (1941).
2. Twombly, G. H., *Endocrinology*, **20**, 311 (1936).
3. Zondek, B., Solman, F., and Hochman, A., *Proc Soc Exp Biol and Med*, **39**, 283 (1938).
4. Cacioppo, F., and Fazio, F., *Boll Soc ital. biol sper*, **13**, 953 (1938).

URINARY METABOLITES OF SODIUM SALICYLATE*

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Salicylates have been used empirically in the treatment of rheumatic fever for half a century; yet the mechanism underlying their therapeutic effects is still obscure. We have sought to identify the metabolic products in the urine following salicylate administration to human subjects. In this work existing methods of estimation had to be considerably modified for routine analytical purposes. The present paper deals with the identification and amounts of salicyl derivatives occurring in the urine of human subjects taking sodium salicylate, including individuals free of infection and rheumatic subjects with active disease.

EXPERIMENTAL

No dietary restrictions were imposed on the subjects. Urine specimens were collected without preservative, kept at room temperature, and delivered to the laboratory within 24 hours of collection. Specimens which had to be kept for longer periods were refrigerated.

*Analytical Procedure for Total Salicyl*¹—10 ml. of whole or diluted urine plus 3 ml. of concentrated HCl are boiled under a reflux for 3 hours. The hydrolysate is cooled and extracted with ether for 1 hour in the continuous extraction apparatus of Quick (1). To the ether extract are added 3 ml. of N NaHCO₃ and about 5 ml. of water. The ether is evaporated and the aqueous residue boiled for about 5 minutes in order to drive off any volatile phenols and other neutral products which might interfere with the bromination procedure. The hot solution is weakly acidified with sulfuric acid and diluted to about 35 ml. The bromine consumption² is then determined

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The data in this paper are taken from a thesis submitted by Eleanor M. Kapp in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University.

¹ The term "salicyl" will be used throughout this paper to refer to the *o*-hydroxybenzoyl group, which may occur either as free salicylic acid or as some conjugated product.

² Small amounts of ether-soluble bromine-absorbing material were found in the urines collected before medication, and in a control series (about twenty-four specimens) from unmedicated subjects. It was assumed that equal amounts of similar non-salicyl matter would be present in ether extracts from all specimens.

according to the procedure of Quick (2); 10 ml. of 0.2 N bromate are ordinarily enough.

The specificity of the above analysis is greatly enhanced by extraction of the salicylic acid with petroleum ether (boiling range 37–40°). Negligible amounts of adventitious bromine-absorbing matter are carried over into such extracts. However, as this procedure requires 18 to 24 hours instead of 1 hour, its routine use was limited to the estimation of salicylate excreted as such (described below) and in certain other experiments where a high degree of specificity was required.

Fractionation of Urine—To 15 ml. of urine in an extraction cylinder are added 0.5 ml. of 5 N H_2SO_4 and enough 10 per cent sodium tungstate to precipitate all foam-producing substances. 2 drops suffice for a normal urine, but specimens from patients with fever sometimes require as much as 20 drops (and additional acid to keep the final pH below 3). The mixture is extracted continuously with ether for 90 minutes. The extract is designated E and the aqueous residue R.

The extract E is freed of ether, taken up in water, acidified with sulfuric acid to pH 3 or lower and extracted continuously with petroleum ether (boiling range 37–40°) for at least 18 hours. The extract (F) is treated with sodium bicarbonate, boiled, neutralized, and brominated as described under the procedure for total salicyl. The petroleum ether-insoluble residue (G) is reduced in volume to 10 ml. and boiled for 3 hours under a reflux with 3 ml. of concentrated HCl. The resulting hydrolysate is then treated exactly like the hydrolysate in the procedure for total salicyl. The ether-insoluble residue from this last extraction is evaporated to dryness and used for the estimation of glycine.

The residue R is freed of dissolved ether by cautious warming and stirring in a water bath at 60°. The salicylic acid combined in it may be separated by either of the following methods: (a) Hydrolysis of whole residue by addition of 3 ml. of concentrated HCl and heating in a steam bath for 40 minutes. The procedure thereafter is the same as that outlined for total salicyl. When ether is employed for the final extraction, the results are designated R; values obtained with petroleum ether are designated R'. (b) Conjugated material is separated from unhydrolyzed residue R by extracting four times with 10 ml. portions of butanol. The combined extracts are freed completely of solvent by vacuum distillation, and the gummy residual matter is taken up in water, filtered, and made up to 25 ml. Aliquots of the resulting solution are then hydrolyzed and analyzed for salicyl by procedure (a) above, ether being used for the final extraction. Procedure (b) yielded values which agreed well with those referred to as R'.

Estimation of Glycine—This was carried out by Quick's procedure (1) modified by the use of brom-thymol blue alone instead of neutral red and

phenolphthalein. This modification was of considerable advantage, as the solutions were frequently yellow or brownish even after treatment with charcoal.

Isolation and Properties of Salicyl Derivatives

Salicylic acid was identified by melting point (156–157°) and color reactions.

Salicyluric acid was isolated by the procedure of Quick (3) from the urine of a normal subject who had taken 3.5 gm. of sodium salicylate by mouth, in divided doses, on each of 2 successive days. After several recrystallizations from water, two such preparations contained 6.79 and 7.38 per cent of N (theory 7.18), melted at 168.5° and 170.5°, and did not depress the melting point (168.5°) of a synthetic sample.

Analytical Separation from Salicylic Acid—A solution of 2.67 mg. (0.0193 mm) of salicylic acid and 3.66 mg. (0.0188 mm) of synthetic salicyluric acid in 20 ml. of water was acidified with sulfuric acid and extracted continuously with petroleum ether (b.p. 37–40°) for 12 hours (Extract 1) and then for 2 hours with ethyl ether (Extract 2).

<i>Extract 1</i> —Br capacity equivalent to 0.0184 mm salicylic acid					
N content	"	"	0.0005	"	salicyluric acid
<i>Extract 2</i> —Br capacity	"	"	0.0200	"	"
N content	"	"	0.0190	"	"

Reaction with Bromine—The bromine consumption of salicyluric acid, reported by Quick (3) as 2Br_2 per mole, was found to be at this level only when bromination was carried out at a reduced temperature. A 10 minute exposure to free bromine at 10° gave quantitative dibromination, whereas at 25°, under conditions suitable for the complete tribromination of salicylic acid, 15 to 20 per cent of the salicyluric acid present was converted to tribromophenol. It was therefore impossible to draw conclusions from the bromine capacity of a mixture of salicylic and salicyluric acids, as the conditions necessary for their quantitative bromination did not coincide.

The reaction of salicyluric acid with bromine was not applicable to the estimation of this substance in unhydrolyzed G fractions, because of the presence of other bromine-absorbing substances which not only raised the titer but also produced unstable end-points in the thiosulfate titration. Figures obtained from the bromine capacity of unhydrolyzed G fractions at 10° (calculated on the basis of 2Br_2 per mole) were consistently higher than those obtained from the bromine capacity at 25° (calculated as 3Br_2 per mole) of the ether-soluble material extracted after hydrolysis. The latter results were much closer to the figures for glycine. For this reason, hydrolysis and extraction with ether were adopted for the routine determination of salicyl in the G fraction.

Titration with Base—Quick (3) reported that 1 equivalent of salicylic acid required 1.24 equivalents of sodium hydroxide to bring it to a phenolphthalein end-point. The enhancement of the dissociation of the phenolic hydroxyl by conjugation of the carboxyl in peptide linkage is shown by the titration curve of salicyluric acid (Fig. 1), which can be titrated as a monobasic acid to an end-point of pH 5.9. Strict diequivalence was obtained with a phenolphthalein end-point by titrating in 95 per cent ethyl alcohol

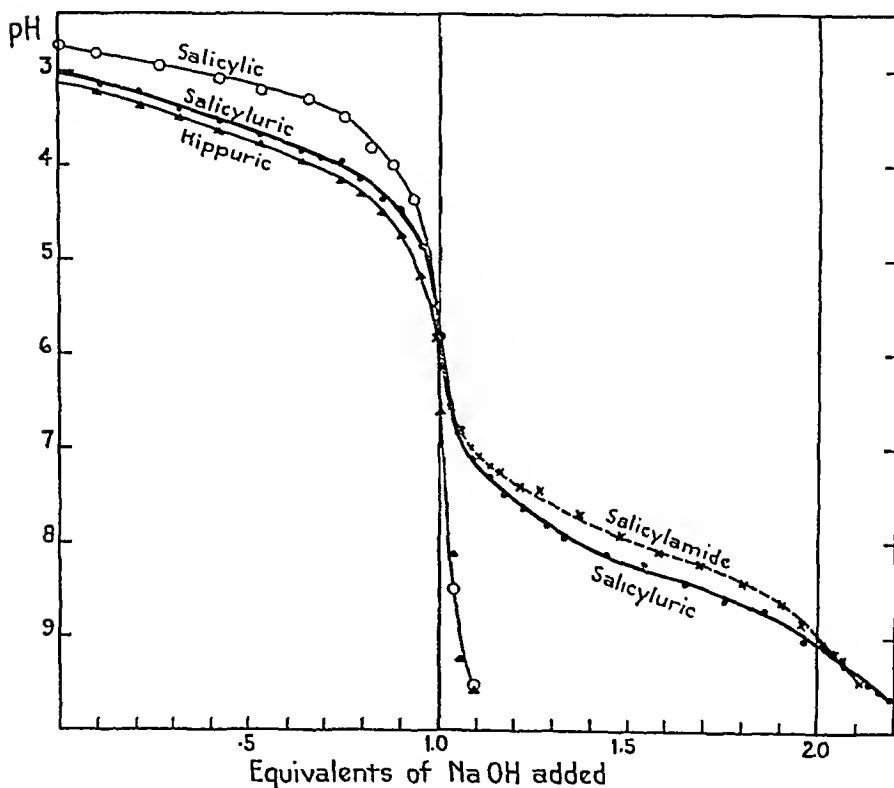


FIG. 1. Titration curves of salicyluric acid and related compounds. 5 ml. of 0.0038 M solution in water titrated with 0.1 M NaOH. The curve for salicylamide is displaced 1 whole equivalent to the right, to facilitate comparison with the analogous part of the salicyluric acid curve.

with barium hydroxide. The insolubility of barium salicylurate allows the phenolic hydroxyl to react to completion. Salicylic acid titrates as a monobasic acid under both conditions.

Titration curves for salicylic acid, salicylamide, and hippuric acid are included for comparison. From the values (Table I) of pK , determined graphically, it can be seen that in salicyluric acid and salicylamide the phenolic group is appreciably ionized within physiological pH limits.

Absorption spectra of salicylic acid were determined at pH levels corresponding to various degrees of ionization of one or both dissociable hydrogen atoms, and are given in Fig. 2. The degree of ionization of the carboxyl hydrogen had no effect on the position of the absorption maximum. A curve made at pH 5.8 (in 0.01 M phosphate buffer) was very similar to the curve for pH 2.0. It had its maximum at 2990 Å., and a slightly lower extinction coefficient. However, complete ionization of the phenolic group (pH 9.4, 0.01 M borate-KCl buffer) involved a shift of the maximum to 3280 Å., and an increase in ϵ from 3680 to 5900. Further increase in pH (material dissolved in excess 0.01 N NaOH) produced no further shift of the maximum. According to the titration curve (Fig. 1), the phenolic group should be about 35 per cent ionized at pH 8, and the absorption spectrum verifies this calculation. The experimental curve obtained at pH 8 is shown in Fig. 2. A few points were calculated for a 65:35 composite of the curves for pH 2 and 9.4, respectively; they lie close to the experimental curve.

TABLE I
Dissociation Constants of Salicylic Acid and Related Compounds

Substance (0.0033 M)	pK, COOH		pK, OH	
	This paper	Other workers	This paper	Other workers
Salicylic acid	3.13	2.99 (4)	10	> 13 (5)
Salicylic acid	3.64		8.16	
Salicylamide			7.95	Acid reaction (6)
Hippuric acid	3.74	3.64 (4)		

Gentisic Acid and Related Compounds—During the recrystallization of samples of urinary salicylic acid it was observed that the aqueous mother liquors gave a transient blue color with ferric chloride. The product responsible for this reaction could be separated from salicylic acid by boiling with hydrochloric acid and successive extraction with petroleum ether and with ether. The ether-soluble material so obtained, on repeated recrystallization from mixtures of ethyl acetate and toluene, yielded a pale yellow product which melted at 198–200°. This, on sublimation in a high vacuum, followed by recrystallization, yielded a colorless product which melted at 202–203° and did not depress the melting point of an authentic specimen (m.p. 202°) of gentisic acid (7).

Analysis— $C_7H_6O_4$. Calculated, C 54.5, H 3.9; found,² C 54.7, H 4.3

The methyl ester had a melting point of 85–86°, unchanged on admixture with an authentic sample (7).

² Microanalyses were obtained through the courtesy of Mr. W. Saschek.

evidence of dissociation of phenolic hydroxyl groups below pH 9.5. Its ultraviolet absorption spectrum in aqueous solution (Fig. 4) shows a maximum at 3225 Å. with a molecular extinction coefficient of 3750 ± 115 , and a strong end-absorption below 2500 Å.; satisfactory coincidence was observed with synthetic and urinary samples.

The reduction of alkaline cupric solutions, carried out according to the Shaffer-Hartmann procedure for blood sugar, was used for assay purposes, and the titration was standardized empirically against pure gentisic acid. In Fig. 5 is shown the relation between millimoles of reducing substance and ml. of 5 mM thiosulfate; and for comparison are included data for hydroquinone and *p*-aminophenol. The reducing power of gentisic acid was

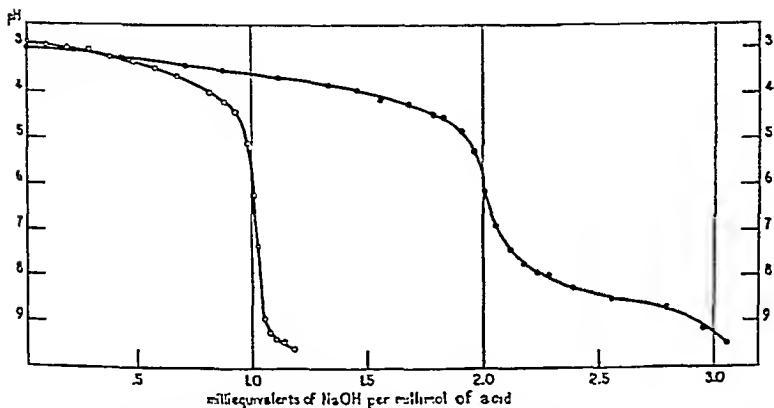


Fig. 3. Titration curves of oxidized derivatives of salicylic acid. O gentisic acid, 0.020 mM in water; • *Uraminsalicylsäure*, 0.009 mM in water.

very similar to that of hydroquinone; *p*-aminophenol was less than half as potent.

Gentisic acid in aqueous solution reacts quantitatively with bromine under almost the same conditions as salicyluric acid. However, in this case the reaction must be carried out at 0°. 4 atoms of bromine are used up per mole of gentisic acid.

The excretion of gentisic acid by human subjects after ingestion of large amounts of salicylate was apparently discovered by Baldoni (8), who did not recognize its identity. Neuberg (9) isolated a similar substance from the urine of dogs which had been given lithium aspirin and recorded his belief that it was gentisic acid. Angelico (10) later confirmed Baldoni's observation and identified the product as gentisic acid.

Another compound giving a transient blue color with ferric salts was

separated by fractional crystallization from aqueous mother liquors of salicyluric acid. After the removal of gentisic acid by recrystallization from mixtures of ethyl acetate and toluene, it melted at 169–170°. It contained nitrogen and reduced alkaline cupric solutions. The color formed with iron salts was deep blue with a purplish tinge, fading to brown, whereas the color given by gentisic acid is a bright blue with no trace of purple, fading to yellow. Only 100 mg. were isolated from the combined etherex-

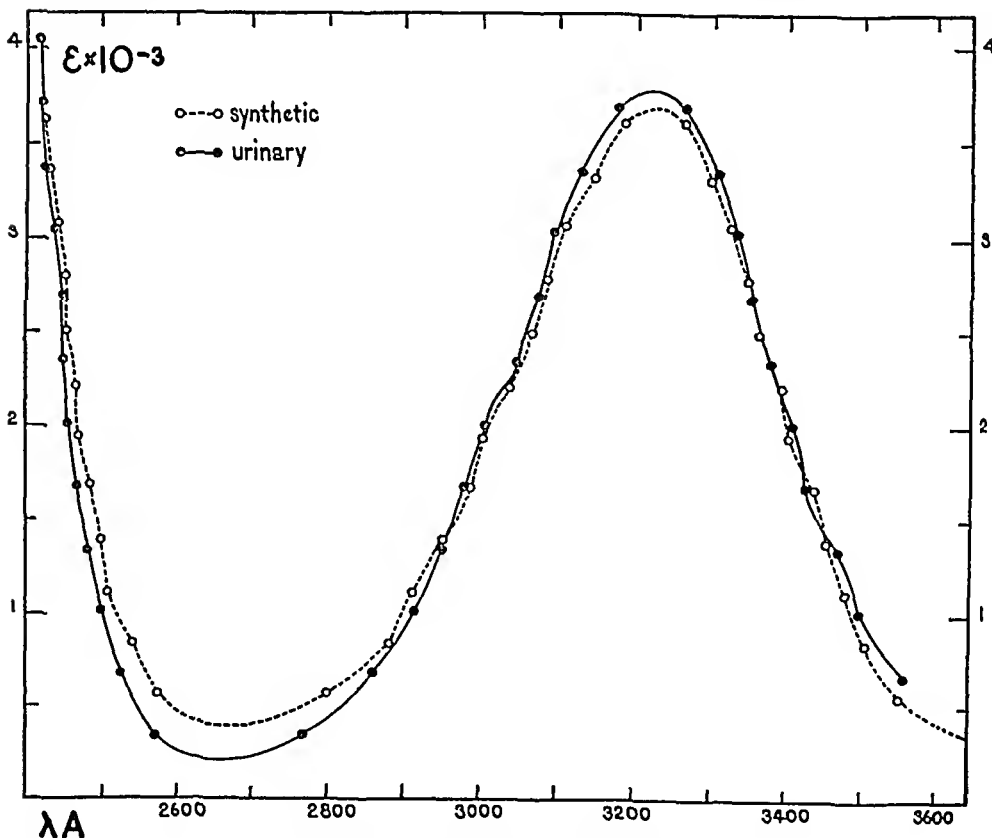


FIG. 4. Absorption spectra of gentisic acid

tracts of several days urine. The behavior of this substance and its composition agreed well with Baldoni's data for *Uraminsalicylsäure*, isolated from the urine of dogs, and there is every reason to suppose that they are the same.

Analysis— $C_{16}H_{13}NO_8$ (347). Calculated. C 55.3, H 3.8, N 4.0
 Found.³ " 55.0, " 4.6, " 4.0

Baldoni's values (8) indicate C 54.3, H 4.8, N 3.7.

A 1.36 mg. sample (0.00392 mM of $C_{15}H_{13}NO_5$) showed by the Shaffer-Hartmann method the same reducing power as 0.0039 mM of gentisic acid. On bromination at 0° for 15 minutes, exactly 4 moles of bromine were consumed per atom of nitrogen. The titration curve (Fig. 3) indicates the presence of one weak (pK 8.3) and two strong (pK 3.8) acid groups per

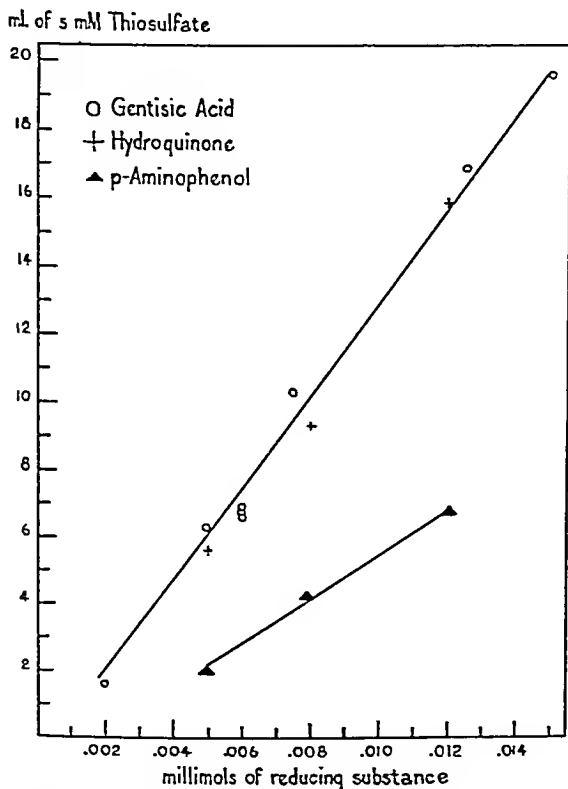


FIG. 5. Calibration curves for the Shaffer-Hartmann titration as applied to the estimation of gentisic acid and related substances.

atom of N. The combined data are in accord with the properties expected for a compound of glycine and salicylic and gentisic acids, such as $HOC_6H_4CO \cdot N(CH_2CO_2H) \cdot C_6H_2(OH)_2COOH$. Lack of material prevented further exploration of this formula.

Salicylglucuronic Acids—Repeated attempts to isolate such substances

have failed. The data to be presented here were secured from fractions obtained by extraction with butanol. These fractions, designated R', were hydrolyzed with 6 per cent HCl on the steam bath for 40 minutes and analyzed for salicyl by the usual technique and for glucuronic acid by a method recently reported (11). The findings (Fig. 6) showed molecular ratios of 1:1 for glucuronic acid to salicylic acid in all four extracts, and indicated that the conjugated derivative could be separated quantitatively from urine by four extractions with butanol.

The qualitative Tollens reaction is given by both glucuronic and galacturonic acids, but their rates of reaction with naphthoresorcinol are quite different (11). The rates of color development of butanol-soluble preparations from four different subjects (Fig. 7) corresponded satisfactorily with the curve for glucuronic acid.

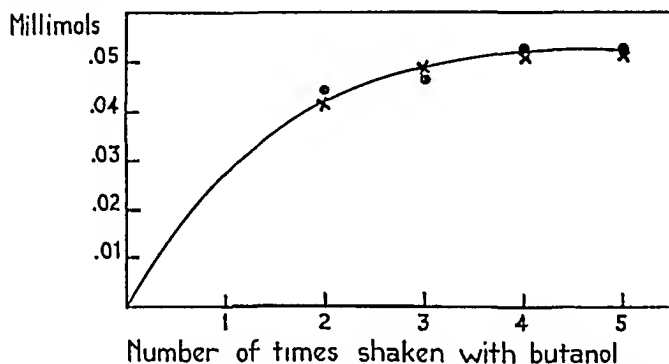


FIG. 6. The course of extraction by butanol of combined salicyl and glucuronic acid from ether-extracted urine. X mm of salicyl in the extract (estimated as free salicyl after hydrolysis); • mm of glucuronic acid in the extract.

Although it has not been found possible to isolate a pure salicylglucuronic acid, data obtained on butanol extracts have furnished suggestions as to the type of linkage involved. A variable proportion, sometimes all, of the salicyl in such fractions was found to be liberated on treatment with alkali. In the few urines with pH 7.4 or higher when delivered to the laboratory, the R fractions were noticeably low in comparison with more acid specimens from the same subjects. The effect was reproduced experimentally in specimens with pH values between 6 and 7 by heating duplicate urine samples with sodium hydroxide prior to the routine fractionation. The salicyl lost from the R fraction by this operation was recovered in the F fraction. And finally, the butanol extract itself was shown to be hydrolyzed by heating at pH 10 to 11. This behavior is analogous to that of benzoylglucuronic acid (12, 13).

In some specimens, part of the butanol-soluble fraction was not alkali-

sensitive but was hydrolyzable by acid. Instances of both types are recorded in Table II. From this it would seem that the conjugation of salicyl with glucuronic acid occurs in more than one way. It is of interest

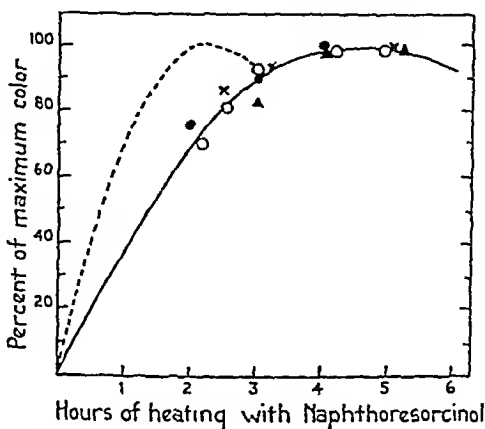


FIG. 7. Color development in relation to time of heating with naphthoresorcinol. The solid curve denotes pure glucuronic acid; the dash curve, pure galacturonic acid. * butanol-soluble material, subject M; X subject S; O subject B; ▲ subject A.

TABLE II

Amounts of Salicyl in Butanol-Soluble Preparations (R') Set Free by Heating in Alkaline and Acid Media

Subject	Salicyl set free by hydrolysis with		Remarks
	NaOH (pH 10-11)	6 per cent HCl	
	mg.	mg.	
CD	12	25	Pool of several preparations of butanol-soluble material
KE	16	13	
WH	25	38	
WJ	69	68	1st 12 hrs. after intravenous medication
	32	57	2nd 12 " " " "
OG	23	38	

that Quick (2) isolated a diglucuronide of *p*-hydroxybenzoic acid, from which 1 glucuronic acid residue could be split by alkali, whereas the other required acid for hydrolysis. It seems probable that a similar dual function of salicylic acid is here involved.

Recovery and Rate of Excretion in Normal Subjects

Brief Courses of Medication by Mouth—2 or 3 gm. of sodium salicylate (equivalent to 60 to 100 mg. of salicyl per kilo of body weight) were administered in 1 gm. doses at 4 hour intervals to several afebrile non-rheumatic children. 24 hour urine samples were collected for 1 day before the dose and for several days thereafter, and analyzed individually for total salicyl content. From 70 to 85 per cent of the drug given was recovered from the urine of normal subjects following a single dose. Not more than

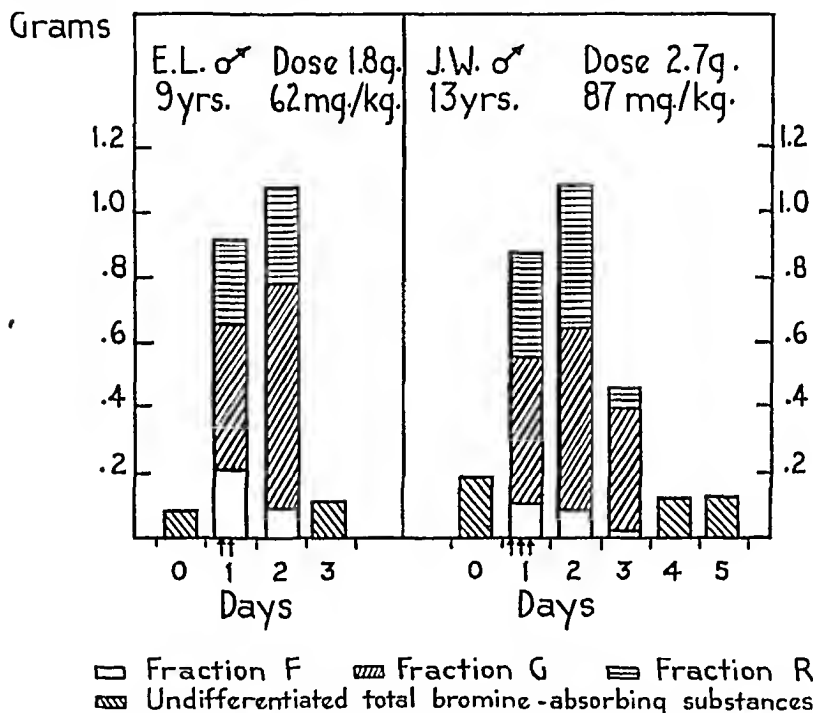


FIG. 8. The daily excretion of salicyl by normal subjects. The arrows indicate the administration of 1 gm. doses of sodium salicylate, equivalent to 0.9 gm. of salicyl.

half the dose was excreted in the first 24 hours (Fig. 8). These observations confirm those of Nencki (14), Hanzlik *et al.* (15), and Holmes (16).

Single Dose, Administered Intravenously—In order to eliminate the delay in absorption after oral administration, a healthy 13 year-old boy was given 1.25 gm. of sodium salicylate intravenously. The urine was collected in two 12 hour specimens for the 1st day, and as 24 hour specimens thereafter. 60 per cent was recovered in 24 hours, distributed equally between the two 12 hour specimens. Little salicyl was obtained in subsequent

collections; the total amount recovered was only 67 per cent of the dose given (Fig. 9).

Repeated Medication for Many Days—The next step was to obtain in normal subjects a fair basis for later comparison with rheumatic patients, who are usually kept under medication for long periods. Two healthy adults engaged in laboratory work were given sodium salicylate daily for 11 days, at a level of 50 mg. of salicyl per kilo of body weight. In each case, about half the daily dose was excreted during the first 24 hours (Fig. 10). Maximal output was attained on the 5th and 2nd days, respectively. The over-all recoveries for the entire period were 78 and 79 per cent.

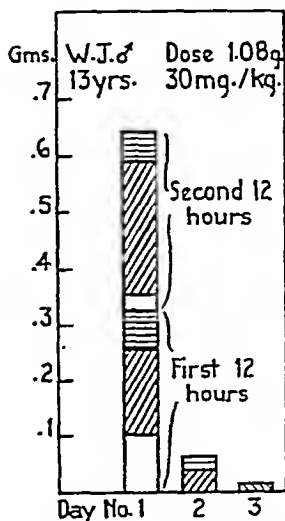


FIG. 9. The excretion of salicyl by a normal subject who was given 1.25 gm. of sodium salicylate (1.08 gm. of salicyl) intravenously. Fractions as indicated on Fig. 8.

Changes in dosage instituted on the 12th day were followed by gradual adjustments in output, over observation periods of 3 days. When medication was stopped, the amount excreted fell off abruptly, which suggested that the drug had not been stored to any significant extent.

Distribution of Salicyl Fractions in Normal Subjects

About one-quarter of the total salicyl was found to consist of free salicylic acid (fraction F, Fig. 10, c and d) and more than half of salicyluric acid (fraction G). These findings are in accord with the data of Holmes (16) and Quick (3).

Fraction R (salicyl not extractable by ether) consisted largely of conjugation products of salicylic acid with glucuronic acid. This accords with the observation (17, 18) that the excretion of conjugated glucuronic acid is increased after the ingestion of salicylates.

Routine analyses for salicyl and glucuronic acid in butanol extracts (R') of normal subjects prepared from daily specimens of urine showed nearly equimolecular ratios (Table III) and indicated (Fig. 10, c and d)

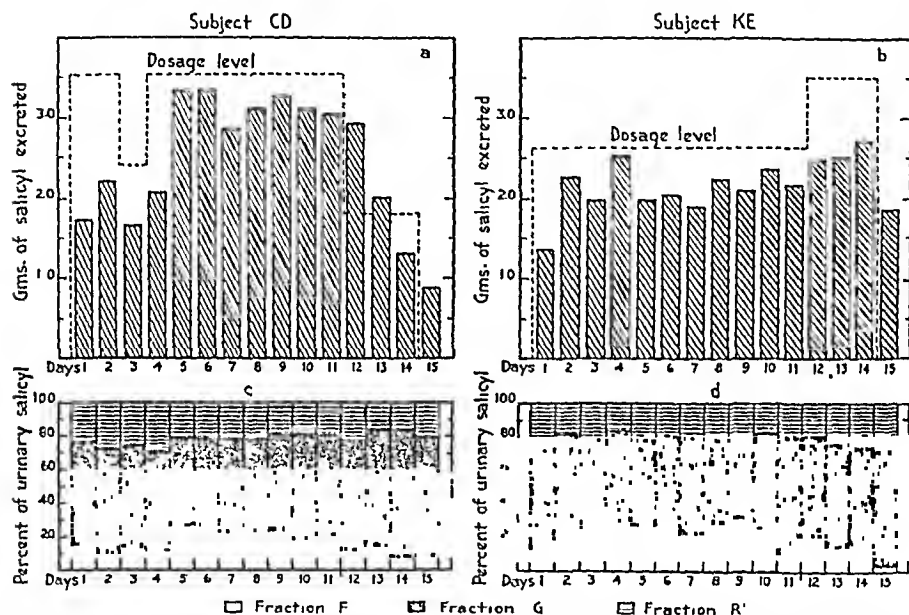


FIG. 10. The excretion of salicyl by normal subjects receiving repeated doses of sodium salicylate over a period of many days. In (a) and (b) is shown the total salicyl recovered in the urine in relation to the dose taken; in (c) and (d), the percentage distribution of the fractions F, G, and R' .

that about 20 to 25 per cent of the total salicyl excreted occurs in this form. This figure is in close agreement with findings of Tollens (17).

Data on the molecular ratios in the G fraction are also given in Table III. In subject KE, the molecular ratios of glycine to salicyl in the G fraction and of glucuronic acid to salicyl in the R fraction were close to 1:1, whereas in subject CD both ratios ran somewhat lower.

The data show the variations to be expected in the several fractions from one day to another in healthy persons on an unrestricted diet, engaged in normal activities. About 80 per cent of the drug taken is accounted for in

(38-40°) excreted 60 to 75 per cent of the dose. Values above 75 per cent were found only by analytical procedures which failed to distinguish between salicyl and dioxy acids. These findings suggested that fever *per se* might have been responsible for the low output. Three patients with fever of non-rheumatic origin were studied; they excreted subnormal amounts (25, 45, and 75 per cent) of salicyl.

Two patients with rheumatic fever were given sodium salicylate intravenously. A 13 year-old boy excreted 41 per cent of a 2.5 gm. dose in 15 hours; a 4 year-old girl excreted 60 per cent of a 2 gm. dose in 48 hours. Both patients excreted more salicyl than would have been expected, even for normal subjects of the same age, after an oral dose. Both showed marked clinical improvement in response to medication.

Patients with acute rheumatism generally excreted a low proportion of salicyl (about 40 per cent) in the form of salicyluric acid; in G fractions both bromine equivalents and glycing to salicyl ratios were below normal (Table III). Relatively large amounts of gentisic acid and *Uraninsalicyl-säure* were excreted, 2 to 4 times as high as with normal subjects, as shown by copper reduction values. The proportions of salicyl excreted as conjugated glucuronates fell within the range observed for normal subjects.

It is a privilege to acknowledge the advice and encouragement of Professor H. T. Clarke throughout this study. We also wish to express our appreciation to Miss Dorothy Childs for technical assistance and to Nurse William Hubbard for her help in the collection of specimens.

SUMMARY

From the urine of human subjects who have taken sodium salicylate four products have been isolated in pure form: salicylic acid, salicyluric acid, gentisic acid, and Baldoni's *Uraninsalicylsäure*. Some evidence as to the constitution of the last named compound has been secured. An appreciable proportion of ingested salicylic acid is excreted in conjugation with glucuronic acid; two varieties of conjugation product appear to occur. Normal adolescents and adults excrete about four-fifths of the ingested salicylate in forms containing intact salicyl groups; about 20 per cent of the excreted salicyl is in unchanged salicylate, about 55 per cent is in salicylurate, and about 25 per cent in glucuronides. About 4 to 8 per cent of the ingested salicyl is converted to gentisic acid and related compounds. Young children excrete considerably lower proportions of ingested salicylate in recognizable forms.

Febile patients excrete a smaller proportion of salicyl than normal subjects of the same age; the output of salicyluric acid is decreased and that of dihydroxy acids is increased.

BIBLIOGRAPHY

1. Quiek, A. J., *J. Biol. Chem.*, 67, 477 (1926).
2. Quiek, A. J., *J. Biol. Chem.*, 97, 403 (1932).
3. Quiek, A. J., *J. Biol. Chem.*, 101, 475 (1933).
4. Ostwald, W., *Z. physik. Chem.*, 3, 369 (1889).
5. Senter, G., and Bulle, F., *J. Chem. Soc.*, 101, 2533 (1912).
6. Limprieht, H., *Ann. Chem.*, 98, 256 (1856).
7. Grabe, C., and Martz, E., *Ann. Chem.*, 340, 213 (1905).
8. Baldoni, A., *Arch. farmacol. sper.*, 8, 174, 193 (1908).
9. Neuberg, C., *Berl. klin. Woch.*, 48, 798 (1911).
10. Angeli, F., *Arch. farmacol. sper.*, 31, 8 (1921).
11. Kapp, E. M., *J. Biol. Chem.*, 134, 143 (1940).
12. Quiek, A. J., *J. Biol. Chem.*, 69, 549 (1926).
13. Goebel, W. F., *J. Biol. Chem.*, 122, 649 (1937-38).
14. Nencki, M., *Arch. Anat. u. Physiol.*, 399 (1870).
15. Hanzlik, P. J., Scott, R. W., and Thoburn, T. W., *J. Pharmacol. and Exp. Ther.*, 9, 247 (1917).
16. Holmes, E. G., *J. Pharmacol. and Exp. Therap.*, 26, 297 (1925).
17. Tollens, C., *Z. physiol. Chem.*, 61, 95 (1909).
18. Salt, H. B., *Biochem. J.*, 29, 2705 (1935).

KETONE-SPARING EFFECT OF GLUCOSE

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From time to time reports have appeared to the effect that animals that are in a state of ketosis show an increase in the excretion of ketone bodies after the administration of carbohydrates. This phenomenon is incompatible with the theory, until recently generally accepted, that carbohydrates exert a ketolytic action in the organism ("Fats burn only in the fire of carbohydrates"). If the theory were valid, carbohydrate feeding could produce only two possible results: it would either decrease ketosis, or, if the organism has completely lost its ability to utilize carbohydrates, ketosis would remain unchanged. An increase of ketosis is incompatible with the theory. Since, however, it does occur, some authors tried to reconcile the fact with their theory by assuming that the glucose "flushes out" and forces into circulation the ketone bodies that have been accumulating in the tissues. This assumption, rather uncritical and arbitrary, is untenable in the light of the work of Harrison and Long (1) who found that in rats the muscle cells are entirely free of ketone bodies until the ketonemia reaches very high levels, and even then the concentration within the cells is much lower than in the blood. As to the liver, these authors have found no higher concentrations of ketone bodies in the intracellular than in the extracellular fluid.

Observations on Diabetic Patients

Our observations on diabetic patients disclosed the fact that an increase in ketonemia and ketonuria, as a result of glucose feeding, is the rule rather than the exception in nearly all cases which show ketonemic levels not lower than 15 to 20 mg. per cent (expressed in terms of β -hydroxybutyric acid). Three examples, presented in Table I, illustrate the phenomenon. The patients in these experiments were fed 100 gm. of glucose about 14 hours after their last meal. Blood samples, obtained directly before and at certain intervals after the administration of glucose, were analyzed for glucose and ketone bodies by methods previously described (2, 3). As the results show, the postabsorptive level throughout the entire period of observation (4 hours). The rise was considerable in all instances; thus, in Case 1 the post-absorptive ketone content of 62.9 mg. per cent was nearly doubled in 4 hours after glucose feeding. Analysis of the urines during these observations

ruled out renal retention as a possible cause of the increase in ketonemia; in Case 3, for instance, the excretion of ketone bodies amounted to 202 mg. during the 1st, and to 539 mg. during the 4th hour.

As pointed out before, this increase in ketosis defies explanation on the basis that glucose exerts a ketolytic action in the human organism. It can be readily explained, however, in the light of extensive and well documented experimental evidence which has been accumulating in recent years.¹ One can postulate on the basis of this evidence that the ketone bodies that are transported in the blood and excreted in the urine are produced in the liver (with some exceptions, perhaps, under extreme conditions). The liver, namely, is unable to burn any appreciable amounts of ketone bodies; hence

TABLE I

Effect of Glucose Feeding upon Ketonemia in Diabetic Patients with Severe Ketosis

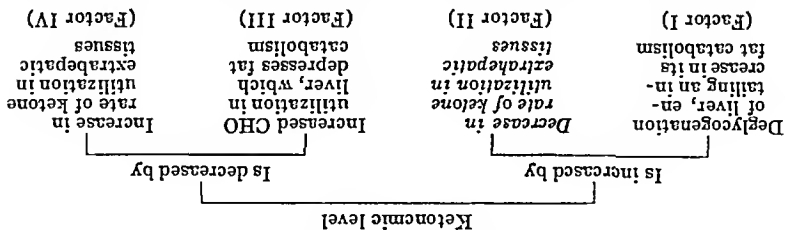
Case No.	Time after glucose feeding	Blood sugar	Acetoacetic acid	β -Hydroxybutyric acid	Total ketone bodies
	hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
1	0	433	21.8	41.1	62.9
	1	575	28.6	59.7	88.3
	2	583	25.7	58.4	84.1
	3	560	34.9	75.5	110.4
	4	548	34.4	81.7	119.1
	0	393	24.9	43.2	68.1
	1.5	514	31.1	50.8	81.9
	3.5	635	37.4	63.9	101.3
2	0	243	7.8	17.6	25.4
	0.5	354	10.5	30.4	40.9
	1	435	10.0	27.5	37.5
	2	503	11.0	22.2	33.2
	3	524	9.2	29.1	38.3
	4	403	13.6	29.5	43.1
3	0	243	7.8	17.6	25.4
	0.5	354	10.5	30.4	40.9
	1	435	10.0	27.5	37.5
	2	503	11.0	22.2	33.2
	3	524	9.2	29.1	38.3
	4	403	13.6	29.5	43.1

when it metabolizes fats (and proteins), it delivers to the blood ketone bodies as the end-products of its fat catabolism. The larger the amount of the fats that are catabolized in the liver, the larger is the amount of ketone bodies passed on to the blood.

Muscle and other extrahepatic tissues, on the contrary, oxidize fat completely, without inhibition at the β -carbon atom stage. In addition, they are capable of oxidizing considerable amounts of ketone bodies that are furnished by the liver (or, for that matter, exogenous ketone bodies injected into the organism). But the capacity of muscle to utilize ketone bodies is¹ Discussion and bibliography concerning the subject may be found in comprehensive articles by Stadie (4), Soskin and Levine (5), and Minsky (6).

limited; therefore, when the rate of hepatic production exceeds the rate of peripheral utilization, hyperketonemia and ketonuria ensue. Thus the ketone body content of the blood represents a balance between the amount of ketone bodies produced in the liver, on the one side, and that utilized in the extrahepatic tissues and excreted in the urine, on the other.

The accompanying diagram based on this concept, presents the known and possible factors which can affect the ketonemic level. It may be noted



that an increase in ketonemia may be due to two factors. The first of these (Factor I), deycogeneration of the liver, invariably augments the rate of production of ketone bodies and, as a consequence, the ketonemic level. This factor can be safely ruled out under the conditions of our observations. As a matter of fact, the increase of hyperglycemia, resulting from glucose feeding, is known to enhance deposition of glycogen in the liver (7). This influence of hyperglycemia is effective also in diabetic conditions, as it has been shown that even the livers of completely depancreatized dogs store glycogen temporarily (8) and at the same time ketone production dimin-ishes (9) at very high glycemic levels.

It is inconceivable, therefore, that glucose feeding should increase the production of ketone bodies in our diabetic subjects. Thus the increase of ketonemia that occurs can be explained only by a decrease of utilization in the extrahepatic tissues (Factor II in the diagram). So long as the muscles have a poor carbohydrate supply (low glycogen reserve), as in the case of the muscle tissues in diabetic subjects, especially in the postabsorptive state, these tissues utilize considerable portions of the ketone bodies that are supplied by the liver. After glucose feeding, however, when glucose reaches the tissues in substantially increased concentrations, the rate of carbohydrate utilization in the muscle cells is greatly enhanced (even in depancreatized animals) and, in consequence, the utilization of ketone bodies is depressed.

When employing the expressions fat-sparing effect, protein-sparing effect of carbohydrate, we recognize the tendency of the mammalian organism to oxidize carbohydrate in preference to other metabolites. In the instance of our observations, when carbohydrate is made available in the muscle tissues

of diabetic subjects, its preferential oxidation leads to the sparing of ketone bodies, a process which manifests itself in an increased ketonemic level. This competition for oxidation was demonstrated also in isolated organs. Edson (10) has shown that in liver slices are oxidized (leading to an increased production of ketone bodies) only when no carbohydrate is available; as soon as carbohydrate enters the metabolic mixture, it is oxidized in preference to fat and, in consequence, the production of ketone bodies is depressed. Waters *et al.* (11) have demonstrated a ketone-sparing effect of glucose in heart-lung preparations.

It is evident that the ketone-sparing effect of glucose in the extrahepatic tissues can manifest itself in increased ketonemia only when the liver utilizes none or only slight quantities of the carbohydrate that is offered to it. For, if the liver is able to utilize appreciable amounts of carbohydrate, the suppression of fat oxidation leads to a decrease in the production of ketone bodies (Factor III), and this change may cancel or outstrip the opposite change in the extrahepatic tissues. We have actually observed such responses to glucose feeding in some cases of diabetic ketosis. In Case 4, for example (Table II), a continuous drop in the ketonemic level has taken place following the 1st hour after glucose feeding, until the postabsorptive ketonemic level of 14.5 mg. per cent has dropped to 5.5 mg. per cent by the end of the 4th hour. In Cases 5 and 6, as it may be noted, the decrease has gone so far as to end in normal ketonemic levels of 0.8 and 0.6 mg. per cent, respectively, within 3 hours after the administration of glucose (the normal range is 0.3 to 0.9 mg. per cent). This response is much the same as that of healthy individuals who developed ketosis by fasting or restriction of carbohydrate intake.

There is, however, no sharp line of demarcation between cases with high and low degrees of ketosis. In Case 8, for instance, glucose feeding caused an increase in ketonemia, although the postabsorptive level was as low as 3.8 mg. per cent. Case 7 was similar in character. Case 12, on the other hand, with a high postabsorptive ketonemic level of 42.8 mg. per cent, responded to glucose feeding as did the milder cases, in that the ketonemia declined after glucose feeding and stayed below the initial level throughout the 4 hours of observation.

Finally, it can scarcely escape attention that in many cases the ketonemic level shows notable fluctuations. It may first drop below the postabsorptive level only to rise subsequently above and again drop below it, as in Cases 10 and 11 (Table II). In other instances one encounters fluctuations but with the ketonemia always staying above the postabsorptive level, as in Case 3 (Table I) and Cases 7 and 12 (Table II). All of these variations can be readily understood by consulting our diagram. Factor III, acting by way of carbohydrate utilization in the liver, is

apparently still effective to some extent in diabetic patients, particularly during high alimentary hyperglycemia. This process tends to decrease ketonemia by inhibiting the production of ketone bodies. Simultaneously Factor II, in the extrahepatic tissues, acts in the opposite direction. The balance between the two factors decides the ketonemic level. Whenever the effect of Factor II is greater than that of Factor III, ketonemia in-

TABLE II

Effect of Glucose Feeding upon Ketonemia in Diabetic Patients with Mild Ketosis
Total ketone bodies are calculated as β -hydroxybutyric acid.

Time after glucose feeding		Case 4		Case 5		Case 6	
hrs	mg per cent	Blood sugar	Ketone bodies	Blood sugar	Ketone bodies	Blood sugar	Ketone bodies
0	286	14.5	308	7.4	175	5.5	5.5
1	335	16.1	438	3.6	282	3.9	3.9
2	475	10.9	454	2.8	335	1.1	1.1
3	503	6.0	405	0.8	323	0.6	0.6
4	417	5.5	355	1.0	333	0.6	0.6
Case 7*		Case 8		Case 9		Case 10	
0	202	12.1	368	3.8	273	11.0	11.0
1	375	19.1	643	3.7	395	7.3	7.3
2	446	20.0	544	4.7	453	4.5	4.5
3	451	15.4	495	4.2	473	4.6	4.6
4	353	18.9	434	4.9	375	4.7	4.7
Case 11		Case 12		Case 13		Case 14	
0	178	4.4	234	7.6	238	42.8	42.8
0.5	263	3.8	337	5.7	432	42.9	42.9
1	343	6.7	367	7.9	483	35.4	35.4
2	380	2.2	364	3.2	527	26.2	26.2
3	408	2.2	329	5.8	397	36.0	36.0
4	328	3.1	301	5.8	312	26.4	26.4

* This patient was the only one among the cases presented in Tables I and II who had been treated with insulin prior to our observations.

creases, and, conversely, when the rate of Factor II lags behind the rate of Factor III, ketonemia decreases. Continued shifts in the relationship between the rates of action of these two factors entail corresponding fluctuations in the ketonemic level. That Factors I and IV could not have had any part in the changes of ketosis under the conditions that prevailed in our experiments, is, we believe, obvious.

Observations on Dogs

Subtotally (about 80 to 90 per cent) depancreatized dogs, as it is known, are able to utilize normal amounts of carbohydrate without developing any symptoms of diabetes. They show neither glycosuria nor ketosis, and their postabsorptive blood sugar level is normal so long as they are kept on a diet consisting of lean meat, bones, and Purina chow. It was found in this laboratory that when such dogs were switched to a diet of 2 parts of meat and 1 part of fat they gained weight and developed glycosuria and ketosis. Postmortem examinations disclosed that the livers had become degenerated and very fat (fat content of from 16 to 20 per cent). These conditions are not produced by a similar dietary régime in dogs in which the pancreas is intact (unpublished observations).

Dogs, after having been made diabetic in this manner, were employed for experiments similar to those we have performed on diabetic patients. The animals were fed by stomach tube 2 gm. of glucose per kilo of body weight, in some instances in the postabsorptive state, in others after 3 to 4 days of fasting, and the changes in the glycemic and ketonemic levels were observed at certain intervals. In Table III it may be seen that a dog, while kept on a normal diet, showed only a moderate deviation from the glucose tolerance of intact animals. As to the ketonemia, the postabsorptive level was within the normal range. After the administration of glucose an initial drop occurred, then a slight rise, but the changes were within the normal range (such changes we have also observed in healthy men). After, however, the dog had been rendered diabetic by fat feeding, glucose feeding caused the ketonemia to rise considerably above the initial level.

In Table IV are presented two experiments on other animals that were rendered glycosuric and ketonuric by meat-fat diets. These dogs were fasted 4 days before the experiment. As may be seen, the changes in the ketonemia of Dog D were quite similar to those in many diabetic men; in the 1st hour after glucose feeding there was a transitory decline, but at the end of the 4th hour an increase of about 60 per cent above the postabsorptive level has occurred. By the end of the 6th hour a further substantial increase has taken place, raising the ketonemia to 3-fold of the postabsorptive level.

In Dog C the relationship between the glycemic and ketonemic levels is more obvious than in any of the other experiments described in this paper. During the first 1.5 hours after glucose feeding carbohydrate utilization in the liver was slight, so that the production of ketone bodies was not appreciably inhibited, while in the extrahepatic tissues carbohydrate began to compete with the ketone bodies for oxidation, thus exerting a ketone-spar-

ing effect. The balance of the two processes was manifested in an increase of the ketonemic level (Factor II > Factor III, according to the diagram). Subsequently, when the blood sugar lingered at the high concentration of 803 mg. per cent, the liver was enabled to store some glycogen and to catabolize increased quantities of carbohydrate, so that the effect of Factor

TABLE III

Effects of Glucose Feeding in Postabsorptive State upon Ketonemia in Subtotally Depancreatized Dogs

2 gm. of glucose per kilo of body weight given by stomach tube.

Time after glucose feeding	10 wks. after operation, on normal diet		4 mos. after operation, 5 weeks on fat-meat diet	
	Blood sugar	Blood ketones	Blood sugar	Blood ketones
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0	95	0.9	242	13.1
1	233	0.4	460	4.3
2	295	0.4	369	11.3
4	158	0.6	310	15.1
6	95	0.7	308	18.1

TABLE IV

Effects of Glucose Feeding upon Ketonemia in Subtotally Depancreatized (Fat-Fed) Dogs, after a Fast of 4 Days

Time after glucose feeding	Dog C*		Dog D†	
	Blood sugar	Blood ketones	Blood sugar	Blood ketones
hrs.	mg. per cent	mg. per cent	mg. per cent	mg. per cent
0.0	278	1.7	239	5.7
0.25	392	1.8		
0.5	545	2.1		
1.0	674	2.2	354	3.4
1.5	752	3.3		
2.0	803	2.6	299	5.6
4.0	803	1.4	263	9.1
6.0	441	6.1	239	15.2

* 4 gm. of glucose per kilo of body weight.
† 2 gm. of glucose per kilo of body weight.

III outstripped the effect of Factor II, a shift that came to expression in a diminished ketonemia. But by 6 hours after glucose feeding, when the blood sugar has dropped steeply, carbohydrate utilization in the liver declined, the effect of Factor III diminished, and once more Factor II outstripped Factor III, resulting in a great increase of the ketonemic level.

SUMMARY

It has been recognized in recent years that carbohydrates exert no ketolytic effect in the organism and that the phrase, "Fats burn only in the fire of carbohydrates," is unfounded. Now experimental evidence is accumulating to demonstrate the contrary; namely, that carbohydrates exert a ketone-sparing effect in various extrahepatic (in the main, muscle) tissues and, as a consequence, inhibit the burning of ketone bodies. This sparing effect manifests itself after glucose feeding in an increase of the existing ketonemia (and ketonuria) of diabetic patients. The rise in ketonemia occurs, however, only in cases in which the ability of the liver to utilize carbohydrates is greatly impaired, so that the liver continues to use fats (and proteins), and hence to produce ketone bodies, at nearly the same rate as before glucose feeding.

Subtotally depancreatized dogs, whose livers had been rendered very fat (16 to 20 per cent fat content) by protracted administration of high fat diets, develop glycosuria and ketosis. Such animals respond to glucose feeding with an increase in ketonemia and ketonuria in the same manner as diabetic men.

BIBLIOGRAPHY

1. Harrison, H. C., and Long, C. N. H., *J. Biol. Chem.*, **133**, 209 (1940).
2. Shaffer, P. A., and Somogyi, M., *J. Biol. Chem.*, **100**, 695 (1933).
3. Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, **140**, 5 (1941).
4. Stadie, W. C., *J. Clin. Inv.*, **19**, 843 (1940).
5. Soskin, S., and Levine, R., in Lewis, H. B., *Biological symposia*, Lancaster, 5, 61 (1941).
6. Mitrsky, I. A., *J. Am. Med. Assn.*, **118**, 690 (1942).
7. Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, **85**, 275 (1929-30).
8. Mitrsky, I. A., Heiman, J. D., and Broth-Kahn, R. H., *J. Physiol.*, **118**, 290 (1937).
9. Bodo, R. C., Co Tri, F., and Farber, L., *J. Physiol.*, **103**, 18 (1933).
10. Edson, N. L., *Biochem. J.*, **30**, 1862 (1936).
11. Winters, E. T., Fletcher, J. P., and Mitrsky, I. A., *J. Physiol.*, **122**, 512 (1938).

We have encountered cases, however, in which the ketonemia, after an initial dip, has risen well above the postabsorptive level during the 4th or 5th hour interval after the administration of glucose. In some instances a substantial increase appeared only during the 6th hour. Such changes occurred exclusively in subjects in which the alimentary hypoglycemia was followed by variable degrees of hypoglycemia. In Case I (Table II),

above it either at this time or an hour or two later. We have encountered cases, however, in which the ketonemia, after an initial dip, has risen well above the postabsorptive level during the 4th or 5th hour interval after the administration of glucose. In some instances a substantial increase appeared only during the 6th hour. Such changes occurred exclusively in subjects in which the alimentary hypoglycemia was followed by variable degrees of hypoglycemia. In Case I (Table II),

Results so obtained are presented in Table I. They show that the ketonemic level decreases by 30 and occasionally by more than 50 per cent, at one time or another, within 3 hours after the administration of glucose. (We have encountered but few cases, in which the very low postabsorptive ketonemic level has undergone no detectable change, as, for example, in the last two subjects in Table I.) In the 4th hour the ketonemia begins to increase and approaches the postabsorptive level, but does not rise above it either at this time or an hour or two later. We have encountered cases, however, in which the ketonemia, after an initial dip, has risen well above the postabsorptive level during the 4th or 5th hour interval after the administration of glucose. In some instances a substantial increase appeared only during the 6th hour. Such changes occurred exclusively in subjects in which the alimentary hypoglycemia was followed by variable degrees of hypoglycemia. In Case I (Table II),

Normal human blood always contains β -hydroxybutyric acid and acetoacetic acid; the sum of the two acids, expressed in terms of β -hydroxybutyric acid, is less than 1 mg. per cent, and amounts on an average to 0.5 mg. per cent (1). The blood samples which yielded these values were drawn in the postabsorptive state (before breakfast), while blood samples obtained between meals tended to show lower ketonemic levels. This observation prompted a study of the possible changes in the ketonemia of healthy individuals after the administration of 100 gm. of glucose.

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BY MICHAEL SOMOGYI

HEALTHY MAN

EFFECTS OF GLUCOSE FEEDING UPON THE KETONEMIA IN

for instance, as the blood sugar has risen to high levels during the 1st hour after the administration of glucose, the ketone bodies dropped by 45 per cent below the postabsorptive level. In the 3rd and 4th hours, however, when the blood sugar receded to the low level of 50 mg. per cent, ketonemia increased and exceeded the lowest previous level by 272 per cent, and the postabsorptive level by 104 per cent. Cases 2, 3, and 4 show changes of the same character to an even more pronounced degree. The observations on the last two cases given in Table II were made on healthy students after 3 days of fasting. As may be noted, in Case 5

TABLE I

Showing That Postabsorptive Ketonemic Level of Healthy Individuals Declines within 3 Hours after Glucose Feeding

The ketone bodies are calculated as mg. of β -hydroxybutyric acid per 100 cc. of blood.

Subject					Time after administration of glucose				
					0 hr.	1 hr.	2 hrs.	3 hrs.	4 hrs.
L. M., 13 yrs., M.	0.51	0.51	0.66	0.73	0.51	0.36	0.58	0.36	0.47
A. B., 51	"	"	0.66	0.73	0.66	0.36	0.35	0.26	0.50
L. W., 37	"	"	0.73	0.73	0.73	0.24	0.39	0.26	0.37
H. G., 45	"	"	0.16	0.16	0.16	0.12	0.38	0.26	0.46
A. D., 20	"	"	0.52	0.52	0.52		0.16		
A. S., 59	"	"	0.58	0.58	0.58		0.16	0.37	
C. H., 23	"	"	0.28	0.28	0.28	0.16	0.16	0.14	
E. G., 22	"	"	0.84	0.84	0.84	0.65	0.44	0.68	
A. B., 38	"	"	0.60	0.60	0.60	0.40	0.44		
E. M., 48	"	"	0.73	0.73	0.73	0.40	0.41		
E. H., 36	"	"	0.36	0.36	0.36	0.40	0.23		
S. B., 11	"	"	0.81	0.81	0.81	0.48	0.23		
G. W., 26	"	"	0.77	0.77	0.77	0.19	0.23	0.22	0.67
E. K., 19	"	"	0.27	0.27	0.27	0.23	0.23		0.24
S. S., 51	"	"	0.25	0.25	0.25	0.32	0.21		0.28

ketonemia rapidly and consistently decreased after glucose feeding until, in 4 hours, it dropped from the initial 38.1 mg. per cent to the near normal level of 1.9 mg. per cent. The response in Case 6 was different. Ketonemia declined during the first 3 hours much in the same manner as in the preceding case, but in the 4th hour it has increased from 2.5 mg. per cent to 10.8 mg. per cent, an increment of 332 per cent. As may be noted, this change coincided with a drop of the blood sugar to a rather low hypoglycemic level.

We offer the following interpretation of the facts described. The initial decrease in ketonemia is due to the fat- and protein-sparing effect of carbohydrate.

greater is the rise in the ketonemic level. In support of this view we point to Case 4 in Table II, in which the hypoglycemia was more intensive than in the other three examples, and it soon was followed by a substantial increase of the blood sugar. After 4 hours had elapsed since the administration of glucose, this increment in the glucose content of the blood can be accounted for only by hepatic glycogenolysis. From a comparison of the blood sugar changes in Cases 1, 2, 3, and 4, it is evident that the increase in the rate of hepatic glycogenolysis was distinctly higher in Case 4 than in Cases 1, 2, and 3. Concurrently, the increase in ketonemia also was greater; it represents an elevation of 803 per cent above the post-absorptive, and of 1300 per cent above the lowest (2 hour) ketonemic level.

It is generally assumed that the production of ketone bodies is increased only when the glycogen stores of the liver have suffered a far reaching depletion. Such a condition can be safely ruled out in our cases in which normally nourished individuals had consumed 100 gm. of glucose only 4 or 5 hours before the ketonemic level has shown a marked rise. It may be stated, as was pointed out in a previous study in hyperpyrexia (5), that an increase in the rate of hepatic glycogenolysis alone seems to suffice to increase the rate of ketone body production, notwithstanding the amount of glycogen present in the liver. This interpretation of our findings is strengthened by the work of Hubbard and Wright (6) who observed a rise in ketonemia after the administration of small doses of adrenalin to normally nourished men, directly after a meal.

Indirect evidence in favor of our view is furnished by experiments which show that ketogenic factors fail to act as such if their glycogenolytic effect is counteracted. Hirschhorn and Pollak (7) found this to be the case in the instance of adrenalin. They reported in 1927 that the ketonuria of phlorhizinized rabbits was markedly increased by the injection of adrenalin; but the simultaneous injection of ergotoxine, a drug known to block in the liver the glycogenolytic effect of adrenalin, also prevented its ketogenic action. Mirsky (8) made similar observations when he used anterior pituitary lobe extract as the glycogenolytic-ketogenic factor. Fasted rabbits developed considerable degrees of ketosis when injected with the extract; but the simultaneous injection of ergotamine or of insulin, both of which inhibit hepatic glycogenolysis, nullified the ketogenic effect. A third observation to the same effect was made in this laboratory, under entirely different conditions. We have shown that the consistent ketogenic effect of artificial fever, which follows the increase in the rate of hepatic glycogenolysis, can be completely prevented by continuous injections of glucose at a rate that suffices to maintain hyperglycemic states (5). Hyperglycemia, as is known, depresses hepatic glycogenolysis.

SUMMARY

In healthy individuals a measurable decrease of the ketonemic level takes place within from 1 to 3 hours after the administration of glucose. This change is attributed to the fat- and protein-sparing effect of carbohydrate in the metabolism of the liver.

In cases in which the alimentary hyperglycemia is followed by hypoglycemia (usually during the 3rd or 4th hour after the administration of glucose) the ketonemic level rises considerably above the postabsorptive level; this rise lags behind the appearance of hypoglycemia and occurs in the 4th or 5th hour, sometimes only in the 6th hour. The explanation is offered that an increase in the rate of hepatic glycogenolysis entails an increase in the rate of production of ketone bodies, regardless of the nature of the glycogenolytic factor. Thus, spontaneous hypoglycemia, protracted hypoglycemia caused by insulin, fever, adrenalin, anterior pituitary extract, all produce increased ketonemia at the same time that they enhance the rate of hepatic glycogenolysis. They fail, however, to increase ketosis if, simultaneously with them, factors are at work which inhibit hepatic glycogenolysis.

BIBLIOGRAPHY

1. Weichselbaum, T. E., and Somogyi, M., *J. Biol. Chem.*, **140**, 5 (1941).
2. Edson, N. L., *Biochem. J.*, **30**, 1862 (1936).
3. Somogyi, M., *Proc. Soc. Exp. Biol. and Med.*, **45**, 644 (1940).
4. Somogyi, M., *J. Biol. Chem.*, **141**, 219 (1941).
5. Somogyi, M., and Kirstein, M. B., *Proc. Soc. Exp. Biol. and Med.*, **45**, 640 (1940).
6. Hubbard, R. S., and Wright, F. R., *J. Biol. Chem.*, **49**, 385 (1921).
7. Hirschhorn, S., and Pollak, L., *Z. klin. Med.*, **105**, 371 (1927).
8. Mirsky, I. A., *Am. J. Physiol.*, **116**, 322 (1936).

THE MECHANISM OF THE EFFECT OF CALCIUM SALTS ON THE SUCCINOXIDASE SYSTEM*

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In a preliminary report (1) we described a strong stimulation of the oxidation of succinate by fresh tissues when traces of calcium salts were added. After a number of preliminary studies to determine the conditions under which the calcium effect may be observed, the following working hypotheses were developed to explain the stimulatory effect of calcium on the succinoxidase system. (1) Calcium, by its well known influence on membrane permeability, in some manner causes the rate of succinate oxidation to increase. (2) The calcium ion might remove some inhibitory substance, either one formed by the reaction or one otherwise present in the tissue. This effect might be due either to combination with the inhibitor or to the catalytic removal of the inhibitor. (3) The calcium ion might be a specific activator for succinic dehydrogenase, or some other component of the succinoxidase system. This activation could be accomplished in either of two ways: Calcium could become an integral part of the enzyme, necessary for its activity; or, calcium could be catalytically involved in the activation of the enzyme. (4) The calcium ion might prevent the formation of some inhibitory substance.

The experiments reported in this paper show that calcium functions by preventing the formation of oxalacetate, a strong inhibitor for the succinoxidase system.

Methods

Respiratory studies were conducted, for the most part, in Barcroft differential manometers (2). Details of the additions to the various flasks will be given with each experiment reported. Air was used in the gas phase and potassium hydroxide placed in the center cups in each experiment. All succinoxidase experiments were equilibrated 10 minutes before the cocks were closed. The bath temperature was 37°, and the flasks were shaken at about 100 cycles per minute. Results are expressed as Q_{O_2} (microliters of oxygen consumed per mg. of dry weight of tissue per hour).

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The Q_{O_2} values given are the maximum values observed in any of the 10 or 15 minute periods between readings.

Tissues were obtained by decapitating white rats and removing the required tissue immediately. The sample was blotted on a piece of moist filter paper to remove blood and excess interstitial fluid. If it was to be minced, the sample was run through a Seevers and Shideman (3) mincer and portions of the mince weighed on slips of cellophane and placed in the flasks. A tissue to be homogenized was weighed in a homogenizer tube, the appropriate amount of water or buffer added, and the tissue homogenized by the Potter and Elvehjem (4) technique. Firm tissues, such as heart or skeletal muscle, were minced before being weighed into the homogenizer tube. Aliquots of the homogenate were added by pipette to the manometer flasks or other reaction vessels.

Calcium analyses were performed by the dry ashing method, by precipitating the calcium as tricalcium phosphate, and determining the phosphate in the precipitate by the Fiske and Subbarow method (5).

Cozymase assays were conducted by the method of Axelrod and Elvehjem (6), modified to suit the particular apozymase preparation used, since for maximum carbon dioxide production, this apozymase required, in addition to the usual reagents, also muscle adenylic acid and cocarboxylase. An assay flask would thus contain Mg and Mn (1 mg. each, as chlorides, per ml.) 0.10 ml., glucose (40 per cent in 0.1 M phosphate buffer, pH 6.2) 0.20 ml., buffer (1 M phosphate, pH 6.2) 0.06 ml., hexose diphosphate (10 mg. of organic P per ml.) 0.4 ml., muscle adenylic acid (1 mg. per ml.) 0.2 ml., cocarboxylase (1 mg. per ml.) 0.02 ml., water 0.02 ml., sample plus water 1.0 ml., apozymase 50 mg.

Reagents other than those used in the cozymase assay were prepared as nearly calcium-free as possible, and analyses of them showed no detectable calcium.

EXPERIMENTAL

To determine the correctness of hypothesis (1), a comparison of the effect of calcium in minced and homogenized tissues was made. In the mince, only a portion of the cells is ruptured, while in a carefully prepared homogenate, almost all of them are destroyed. The results are given in Table I.

It is thus apparent that though the homogenized tissues give much higher rates of respiration than do the minced tissues, the calcium effect is present in both types and therefore is not dependent upon a permeability effect.

To test hypothesis (2), it was decided to see if the presence of calcium relieved the inhibition due to any of the known inhibitors of succinoxidase which might be present in the system. The three inhibitors tested were

TABLE I

Comparison of Calcium Effect on Minced and Homogenized Tissues

Buffer, M/60 Na and K phosphate, pH 7.4; substrate, 90 micromoles of sodium succinate per flask; tissue, 50 mg. of rat liver per flask; total volume, 2.0 ml.

Flask No.	Preparation of tissue	Calcium chloride added	QO ₂
		<i>micromole</i>	
1	Minced	0.025	19.4
2	"	0.125	24.4
3	"	0.250	24.5
4	Homogenized	0.025	43.9
5	"	0.125	52.1
6	"	0.250	53.7

TABLE II

*Effect of Calcium on Inhibition of Succinoxidase System by Fumarate, Malate, and Oxalacetate*Buffer, M/45 Na and K phosphate, pH 7.4; cytochrome c, 0.02 micromole per flask; substrate, 90 micromoles of sodium succinate per flask (tipped from Keilin cups after the 10 minute equilibration period in order to measure the true initial QO₂); tissue, 20 mg. of homogenized rat heart.

Experiment	Flask No.	Inhibitor	Calcium chloride	QO ₂
		<i>micromoles</i>	<i>micromoles</i>	
A	1	None	0.0	40.5
	2	"	0.5	120.5
	3	5 fumarate	0.0	17
	4	5 "	0.5	83
	5	5 malate	0.0	17.5
	6	5 "	0.5	86
B	1	None	0.0	40.5
	2	"	0.5	127
	3	1.0 oxalacetate	0.0	5
	4	1.0 "	0.5	2.5
	5	0.1 "	0.0	29
	6	0.1 "	0.5	99
C	1	None	0.0	68.5
	2	"	0.5	80
	3	"	2.5	78.5
	4	0.2 oxalacetate	0.0	28.5
	5	0.2 "	0.5	27.5
	6	0.2 "	2.5	31

malate, fumarate, and oxalacetate, as illustrated in Experiments A and B, Table II. Oxalacetate was prepared from the commercial sodium ethyl oxalacetate (7).

These experiments are not conclusive. Although it is true that the calcium level used was insufficient to overcome the effect of the added inhibitor, it is possible that higher levels of calcium might do so, if the calcium actually combines with the inhibitor. Experiment C (Table II) tests this point.

The fact is here demonstrated that the use of even 10 times as much calcium as oxalacetate does not relieve the oxalacetate inhibition at all. Oxalacetate was chosen for this experiment, rather than fumarate or malate, because it is a very potent inhibitor of the succinoxidase system; so that very small amounts, of the same order as the amounts of calcium used, gave inhibitions of the proper magnitude to compare with the inhibition produced by omission of calcium from the system. We may conclude that calcium does not act by removing any of these compounds as inhibitors.

TABLE III

Variations in Time Relationships of Substrate and Calcium Additions

Buffer, $M/45$ Na and K phosphate buffer, pH 7.4; cytochrome *c*, 0.02 micromole per flask; tissue, 20 mg. of homogenized rat heart per flask; substrate, 90 micromoles of sodium succinate per flask; calcium chloride (when used), 0.05 micromole per flask.

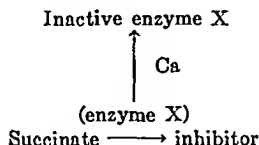
Flask No.	Time relationships	CO ₂
1	Ca and succinate added at beginning of equilibration period	77.5
2	" " added at beginning of equilibration and succinate added 20 min. after closing cocks	152
3	Succinate added at beginning of equilibration and Ca added 20 min. after closing cocks	14.5
4	Succinate and Ca both added 20 min. after closing cocks	91
5	" " added at start of equilibration; no Ca added	15
6	" " 20 min. after closing cocks; no Ca added	68

Assuming for the moment that hypothesis (2) is ruled out, it should be possible to distinguish between hypotheses (3) and (4) by an experiment in which the time of adding the calcium to the system is varied.

If calcium acts as a specific activator for some component of the succinoxidase system, a reaction which is proceeding slowly because calcium has been omitted should be accelerated on the addition of calcium. On the other hand, if calcium acts only by preventing the formation of an inhibitory substance, the reaction taking place in the absence of calcium will already have formed the inhibitory substance and the subsequent addition of calcium will have no stimulatory action. The next experiment was designed to distinguish between these two modes of action of the calcium ion and the results are given in Table III.

These data are entirely in support of hypothesis (4), in that calcium

has no effect when added after the reaction has proceeded for 30 minutes (compare Flasks 3 and 5). Calcium therefore cannot be an activator for the succinoxidase enzymes. In addition, the oxygen uptake is much greater if the calcium chloride is added before the succinate than it is if the two are added simultaneously (compare Flasks 2 and 5). This would indicate that the inhibitory material is formed from the succinate by a system which is inactivated by calcium.



When the succinate is added at the same time as the calcium, the inhibitory material is formed from it before the calcium has had a chance to inactivate the system forming it, while, if the calcium is added to the system before any of the succinate, the inhibitor-forming system is inactivated before any inhibitor is formed. Flask 6 shows that this inactivation of the inhibitor-forming system takes place without the addition of calcium but at a much slower rate. Elliott and Greig (8) found similarly an increase in the succinoxidase activity of tissues stored in the refrigerator.

From these data it appeared probable that the inhibitor sought would be one which is a product of succinate metabolism and the formation of which is conducted by a system which is spontaneously inactivated in the tissue preparations, but which is more rapidly inactivated in the presence of calcium ions.

Of the known inhibitors for the succinoxidase system, the only ones which are breakdown products of succinate are fumarate, malate, and oxalacetate. Fumarate may be eliminated from consideration because it must of necessity be formed by the oxidation of succinate. Malate, being no more inhibitory than the fumarate from which it is derived, could not be the inhibitor sought. Oxalacetate, however, is a very much more potent inhibitor than fumarate or malate; so if a small amount of oxalacetate were formed, we might expect a considerable inhibition of the succinoxidase system. Oxalacetate is formed from malate by a malic dehydrogenase linked with coenzyme I (cozymase). It is well known that cozymase rapidly disappears from minced tissues (9-12); so if the cozymase is destroyed, no oxalacetate should be formed, and the succinoxidase system should not be inhibited. On this basis it is postulated that *the calcium effect is one of accelerating the destruction of cozymase in the tissues so that oxalacetate may not be formed to inhibit the succinoxidase system.*

This hypothesis was tested against all the data accumulated to date and

found to fit perfectly. The results given in Table IV, which show that calcium *inhibited* the oxidation of lactate, pyruvate, glucose, and the endogenous substrates, give particular support to this view. This result would be expected, since cozymase is involved in all of these respirations.

TABLE IV

Effect of Calcium Chloride on Oxidation of Various Substrates

Buffer, M/30 Na and K phosphate, pH 7.4; tissue, 50 mg. of minced liver per flask (minced, rather than homogenized tissues were employed because the homogenization reduced almost to zero the power of these tissues to oxidize this group of substrates); substrates, lactate and pyruvate used as the sodium salts.

Flask No.	Substrate	Calcium chloride added	QO ₂
	<i>micromoles</i>	<i>micromole</i>	
1	None		4.2
2	"	0.5	2.15
3	180 dl-lactate		9.9
4	180 "	0.5	6.1
5	90 pyruvate		5.4
6	90 "	0.5	3.2
1	45 glucose		3.7
2	45 "	0.5	1.1

TABLE V

Calcium Chloride and Mixed Lactate and Succinate Systems

Buffer, M/30 Na and K phosphate buffer, pH 7.4; tissue, 50 mg. of minced liver per flask; the substrates were added as the sodium salts.

Flask No.	Substrate	Calcium chloride	QO ₂
	<i>micromoles</i>	<i>micromole</i>	
1	90 dl-lactate		13.8
2	90 "	0.5	8.3
3	90 succinate		15.8
4	90 "	0.5	50.4
5	90 " + 90 dl-lactate		40.3
6	" "	0.5	49.1

Another experiment in agreement with this postulate, if, in some manner, the lactate or its oxidation product, pyruvate, removes the oxalacetate formed, or ties up the cozymase so that it is not available to participate in the oxidation of malate to oxalacetate, is given in Table V.

In the absence of calcium chloride, lactate stimulates the succinoxidase system more than can be accounted for by the respiration of the lactate

itself. On the other hand, in the presence of calcium chloride, lactate has no influence on the succinoxidase system. It appears as if lactate had an effect almost like that of calcium itself. Analysis of the lactate solution showed it to be free of calcium.

Mann and Quastel (12) have studied the system in tissues which destroys cozymase. Our postulate supports their explanation of the inhibition of succinoxidase by cozymase (13) as being a matter of oxalacetate formation. These authors found that nicotinamide would prevent the destruction of cozymase by the enzyme which they have called "cozymase nucleotidase." It was therefore decided to study the effect of nicotinamide on the succinoxidase system. See Table VI.

TABLE VI

Effect of Nicotinamide on Succinoxidase System

Buffer, $M/45$ Na and K phosphate, pH 7.4; cytochrome c, 0.02 micromole per flask; substrate, 90 micromoles of sodium succinate per flask (added after equilibration); tissue, 22 mg. of homogenized heart.

Flask No.	Preparation	Q_{O_2}
1	Tissue homogenized in $CaCl_2$ solution	92.5
2	" " " water; Ca added before equilibration	85
3	" " " nicotinamide solution	41
4	" " " water; nicotinamide added before equilibration	68.5
5	Tissue homogenized in $CaCl_2$; nicotinamide added before equilibration	81.5
6	Tissue homogenized in nicotinamide; $CaCl_2$ added before equilibration	45

When Flasks 3 and 4 are compared, it is apparent that the earlier addition of nicotinamide to Flask 3 protected the cozymase from destruction, lowering the succinoxidase activity.

When Flasks 3 and 6 are compared, the figures show that even though the calcium chloride was added to Flask 6 before the succinate, it had no stimulatory effect on the succinoxidase, because the nicotinamide was protecting the cozymase.

A comparison of Flasks 5 and 6, each of which contained both calcium chloride and nicotinamide, but added in reverse order, shows that in Flask 5 the calcium chloride had largely completed the destruction of the cozymase (*cf.* Flask 2) by the time the nicotinamide was added, while in Flask 6 the cozymase was protected from the action of calcium chloride by the previous addition of nicotinamide.

The final test of this theory thus lies in actually determining the rate of destruction of cozymase in the presence and absence of calcium.

A piece of fresh rat liver was dropped into 10 cc. of redistilled ice water in a homogenizer tube and homogenized quickly. 5.0 ml. samples were transferred to each of the following: (a) 5.0 ml. of water at 30°, and (b) 5.0 ml. of calcium chloride solution (100 γ of calcium per ml.) at 30°. Samples of each of these were withdrawn at noted intervals, brought to a boil, and placed in a boiling water bath for 2 minutes to destroy the cozymase nucleotidase, and then cooled in an ice bath. These extracts were assayed for cozymase by the yeast fermentation method described above. The

TABLE VII
Effect of Calcium on Destruction of Cozymase

Sample taken at	Cozymase remaining	
	No calcium added	Plus added calcium
min.	γ	γ
4	7.7	7.4
10	5.5	4.2
20	4.8	1.5

TABLE VIII
Glutamate and Succinoxidase System

Buffer, M/30 potassium phosphate, pH 7.4; cytochrome c, 0.02 micromole per flask; substrate, 90 micromoles of potassium succinate per flask; tissue, 50 mg. of homogenized heart.

Flask No.	Oxalacetate	Calcium chloride	Glutamate	CO ₂
	micromole	micromole	micromoles	
1				49
2		0.4		73
3	0.2	0.4		35
4	0.2	0.4	6	65
1				47
2		0.4		69
3			6	63
4		0.4	6	89

results are given in Table VII. Each assay represents 36 mg. of fresh liver.

This confirms our theory that the calcium ion stimulates the destruction of cozymase by animal tissues.

The other piece of direct evidence desired to support this theory would be to measure the amounts of oxalacetate formed in the Barcroft flasks in the presence and absence of calcium. Unfortunately, the amounts are too small for any known method of analysis. Approximately 0.2 micromole

of oxalacetate gives the inhibition of the succinoxidase system obtained by the omission of calcium from that system. If this were assayed by measuring the carbon dioxide formed by decarboxylation (14, 15), the amount formed would be only 4.5 microliters, which is not measurable in the Barcroft apparatus with any degree of precision.

However, an indirect bit of evidence for the presence of oxalacetate as the inhibitor involved is given in Table VIII. These experiments were suggested by Dr. V. R. Potter. Oxalacetate, in the presence of glutamate, will transaminate with the latter to give α -ketoglutarate and aspartate (16); so the addition of glutamate should remove at least a portion of the oxalacetate and accelerate the succinoxidase system.

This shows that glutamate will, to a large extent, relieve the inhibition due to added oxalacetate.

Table VIII shows that glutamate is stimulatory to the succinoxidase system, approaching the activity of calcium chloride itself.

DISCUSSION

This entire study has been concerned with the effect of the calcium ion, the other ions which were present in the solution being ignored. To study the influence of a single ion directly is quite impossible, since at least one anion must be added with the cation. Also, buffers are essential to maintain the reactions at more or less constant rates, and the substrate must be added as a salt.

A few studies on the effects of other ions on the cozymase nucleotidase activity and on the succinoxidase activity of tissues were made, but though general correlation existed, the evidence is not sufficiently well developed to be presented here. An interesting paper in this regard is that of Das (9) who reports activation of his cozymase-splitting enzyme by magnesium ion. Elsdon (17) reports a stimulation by magnesium of the succinoxidase activity of minced pigeon breast muscle.

With regard to the stimulatory effect of lactate on the succinoxidase system, a paper by von Euler and Heiwinkel (11) may be of interest. They report that reduced cozymase is much more rapidly destroyed by tissues than is oxidized cozymase. Thus, in the presence of lactate, it is conceivable that the cozymase is reduced through the lactic dehydrogenase and is therefore more quickly destroyed; so that it cannot enter into the conversion of malate to oxalacetate and the succinate system proceeds unimpeded.

Inhibition of respiratory systems by nucleotidases when the respiratory systems require cozymase has been described for the nucleotidase found in snake venoms by Chain (18) and by Mann and Quastel (12). The former author did not find any stimulation for the succinoxidase system by

his snake venom, but he used very high levels which apparently inhibited some component of the succinoxidase by some other mechanism. The inhibition of the cozymase-requiring systems was complete, while that of the succinoxidase system was only partial.

SUMMARY

1. The succinoxidase activity of several minced and homogenized tissues is increased by the addition of traces of calcium chloride to the medium, but the endogenous respiration and respiration in the presence of lactate, pyruvate, and glucose are inhibited by the same concentration of calcium.

2. Lactate, in the absence of added calcium, stimulates the respiration of tissue in the succinoxidase system more than can be accounted for by the respiration of the lactate itself. Lactate has an influence on the succinoxidase system similar to that of calcium.

3. Calcium chloride does not relieve the inhibition of the succinoxidase system caused by adding oxalacetate.

4. Calcium chloride added a few minutes after the addition of succinate to a tissue suspension fails to show any stimulation. Maximum stimulation is secured by adding the calcium a few minutes before the succinate.

5. Tissues standing in the absence of added calcium are gradually activated until they attain the same succinoxidase activity as tissues to which calcium has been added, and these spontaneously activated tissues no longer respond to added calcium.

6. Nicotinamide, added to the tissue a few minutes before the calcium is added, inhibits the succinoxidase system and prevents any stimulation by calcium. If the calcium is added first, the usual stimulation is observed and the nicotinamide has no inhibitory effect.

7. Cozymase is destroyed by macerated tissue more rapidly in the presence of added calcium chloride than in its absence.

8. Glutamate will relieve the inhibition of the succinoxidase system due to oxalacetate and will also stimulate the succinoxidase system to approximately the same extent as does calcium.

9. All of this evidence is in support of the hypothesis that calcium stimulates the succinoxidase system by activating the cozymase nucleotidase present in the tissue so that cozymase is destroyed and cannot function in the dehydrogenation of malate to oxalacetate, which is strongly inhibitory to the succinoxidase system.

BIBLIOGRAPHY

1. Axelrod, A. E., Swingle, K. F., and Elvehjem, C. A., *J. Biol. Chem.*, **140**, 931 (1941).
2. Dixon, M., *Manometric methods*, Cambridge (1934).

3. Seevers, M. H., and Shideman, F. E., *Science*, **94**, 351 (1941).
4. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, **114**, 495 (1936).
5. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
6. Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, **131**, 77 (1939).
7. Simon, L. S., *Compt. rend. Acad.*, **137**, 855 (1903).
8. Elliott, K. A. C., and Greig, M. E., *Biochem. J.*, **32**, 1407 (1938).
9. Das, N. B., *Ark. Kemi, Mineral. o. Geol.*, **13 A**, No. 7 (1938).
10. von Euler, H., and Günther, C., *Z. physiol. Chem.*, **243**, 1 (1936).
11. von Euler, H., and Heiwinkel, H., *Naturwissenschaften*, **25**, 269 (1937).
12. Mann, J. G., and Quastel, J. H., *Biochem. J.*, **35**, 502 (1941).
13. Potter, V. R., *Ark. Kemi, Mineral. o. Geol.*, **13 B**, No. 7 (1939).
14. Edson, N. L., *Biochem. J.*, **29**, 2082 (1935).
15. Ostern, P. Z., *Z. physiol. Chem.*, **218**, 160 (1933).
16. Cohen, P. P., *J. Biol. Chem.*, **136**, 565 (1940).
17. Elsdon, S., *Biochem. J.*, **33**, 1890 (1939).
18. Chain, E., *Biochem. J.*, **33**, 407 (1939).

ULTRACENTRIFUGAL ISOLATION FROM LUNG TISSUE OF A MACROMOLECULAR PROTEIN COMPONENT WITH THROMBOPLASTIC PROPERTIES*

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Recent publications from this laboratory have dealt with the thromboplastic protein: its isolation from beef lungs (2), its behavior towards heparin (3), its chemical composition (4), and its electrophoretic properties (1). In the last paper of this series (1) it was shown that by means of isoelectric precipitation and electrophoretic separation preparations of this lipoprotein were obtained which showed complete electrophoretic homogeneity. On examination in the ultracentrifuge these preparations, however, exhibited heterodispersity which was probably due to the chemical manipulation involved in the isolation of the material. All boundaries observed had high sedimentation constants, indicating the presence of a very heavy protein component.

In the present communication the isolation of the thromboplastic protein by sedimentation in the ultracentrifuge of saline extracts of beef lungs is described. By this method, which avoided manipulation that could lead to a partial destruction of the labile protein, preparations of extremely high thromboplastic potency and satisfactory homogeneity both in the ultracentrifuge and the electrophoresis cell were isolated.

The physical data obtained with a number of preparations are summarized in Table I. The partial specific volume of the protein was very high, $V_{27} = 0.87$, as was to be expected of a protein-lipid complex. The sedimentation constant $s_{20} = 330$ S and the diffusion constant $D_{20} = 0.38 \times 10^{-7}$ (Experiment 5 in Table I) correspond to an enormous molecular weight or, perhaps better, particle weight from rate of sedimentation; viz., 167 million. The calculated frictional ratio $f/f_0 = 1.41$ would give an axial ratio of 8 for a prolate ellipsoid (5), but, since the degree of hydration of the protein is not known, a discussion of the asymmetry relationship appears unprofitable. Because of the low solubility of the protein, the viscosity measurements carried out were not exact enough to permit the calculation of the particle weight from diffusion and viscosity data (6-8). The

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preparations migrated electrophoretically in each case as a single component between pH 7.5 and 8.6. At lower pH values precipitation occurred.

One preparation was subjected to examination in the electron microscope.¹ Several electron micrographs revealed the presence, together with some aggregated material, of a large percentage of almost perfect spheres with a diameter of 80 to 120 m μ .

TABLE I
Physical Data on Thromboplastic Protein Preparations

Experiment No.	Preparation No.	Concentration of protein	pH	s_{20}	$D_{20} \times 10^7$	Particle weight	Electrophoretic mobility ($\mu \times 10^6$)
		<i>per cent</i>		<i>S</i>		<i>millions</i>	
1	I	0.8	8.5	240	0.34	136	8.3
2	II	0.4	8.3	320			
3	III	0.5	8.6	310	0.34	176	
4	"	0.43	8.6	330	0.34	187	
5	"	0.29	8.6	330	0.38	167	
6	"	0.17	8.6	310			
7	IV	0.43	7.5				8.0
8	"	0.43	8.6	350			8.4

TABLE II
Composition of Thromboplastic Protein Preparations

Preparation No.	N	P	N:P ratio
	<i>per cent</i>	<i>per cent</i>	
I			11.6
I-1	8.77	1.29	15.0
I-2	7.50	1.40	11.9
II-1	8.94	1.33	14.9
II-2	8.01	1.47	12.0
III			12.1
IV			11.7
IV-1	7.74	1.43	12.0

Chemical data for a series of preparations are shown in Table II. Analyses of several dry preparations are also included. These were obtained by the concentration in a high vacuum of frozen solutions of the material,

¹ We are highly indebted to Dr. T. F. Anderson of the RCA Manufacturing Company, Inc., Camden, New Jersey, and to Dr. W. M. Stanley of The Rockefeller Institute for Medical Research, Princeton, New Jersey, for the preparation of the electron micrographs.

after ultracentrifugation, electrophoresis, and dialysis. and formed voluminous white felts, which in contrast to previous preparations that had undergone chemical treatment (2) could be readily dispersed in buffer solutions of a pH higher than 7. It will be noted that the phosphorus content of the preparations here discussed was higher and the nitrogen content consequently lower than in material isolated by salt precipitation (1, 2); but the purpose of the present study precluded the use of organic solvents which would have removed part of the phospholipids attached to the protein.

The protein preparations isolated with the aid of the ultracentrifuge had the highest thromboplastic activity yet observed in this laboratory: 0.008 γ could still be easily demonstrated with chicken plasma as substrate (9).

It may be of interest to discuss briefly the possible place of the thromboplastic protein in the theory of blood coagulation. The three main problems, still unsolved, which face the student of this phenomenon are (1) the mechanism of action of vitamin K (*i.e.* the formation of prothrombin), (2) the mechanism of action of the thromboplastic factor (*i.e.* the formation of thrombin), (3) the mechanism of the conversion of fibrinogen to fibrin. The present communication is concerned with the second of these problems.

Most workers are agreed that a factor present in tissues and blood platelets is necessary for the conversion of prothrombin to thrombin under normal conditions. Certain lipid fractions related to "cephalin" also show activity.² The function of calcium ions is more controversial. In very concentrated prothrombin solutions, spontaneous conversion to thrombin has been observed (11).

The mode of action of the thromboplastic factor is a matter of dispute. Bordet (12) considered thrombin as a compound between prothrombin, calcium, and a thromboplastic lipid ("cytozyme"). This view, at least as far as the transfer of phosphorus-containing substances is concerned, appears to be disproved by experiments with radioactive phosphorus as indicator (13). According to Howell (14) the thromboplastic factor combines with a clotting inhibitor, *viz.* heparin, present in circulating blood as heparin-prothrombin complex, and thereby releases the prothrombin. This is not probable, since it has been shown in this laboratory that, although heparin is able to combine with the protein moiety of the thromboplastic protein, the resulting heparin-protein complex retains marked

² It may here be stated that in an examination of the thromboplastic activity of various tissue phosphatide fractions the phosphatidyl serine from beef brain (10) was found entirely inactive. (Some of these preparations were kindly placed at our disposal by Dr. J. Folch of The Rockefeller Institute for Medical Research, New York.) The activity appears to reside in the highly unsaturated, alcohol-soluble "cephalin" fractions. Preparations from pig and beef heart proved particularly active.

anticoagulant activity (3). Other workers consider the thromboplastic factor as an enzyme which in some manner catalyzes the formation of thrombin from prothrombin (15, 16). This view is supported by the observation that trypsin (16, 17) and certain snake venoms (18) likewise are able to bring about this conversion.

The very active preparations of the thromboplastic protein described in this paper appeared suitable for an examination of the possible enzymatic nature of the thromboplastic effect. Until now, no enzymatic activity has been ascribed to the thromboplastic factor, apart from the hypothetical action on prothrombin. The protein preparations isolated by means of ultracentrifugation of lung extracts were tested for phosphatase and for trypsin activity. They were found to have marked phosphatase action, when tested with sodium β -glycerophosphate. The sodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid,³ a compound exhibiting very high vitamin K activity in the organism (19), likewise was dephosphorylated at a similar rate.

The examination of the thromboplastic protein for a trypsin-like action, carried out with benzoylargininamide hydrochloride⁴ as substrate, gave inconclusive results. In view of the thromboplastic action of crystalline trypsin recorded previously (16), it will be important to reexamine this question by a different method. For the time being, it will suffice to point out that highly purified preparations of the thromboplastic protein do possess enzymatic functions, a fact which supports the view that the thromboplastic effect is enzymatic in nature. It is, indeed, not unlikely that the association of certain enzymes and viruses with cell constituents of extremely high molecular weight is of importance for the orientation of biological reactions within the cell. (Compare the discussion of "heavy" cell constituents in (20-24).)

EXPERIMENTAL

Isolation

In a typical experiment, 1 kilo of finely minced beef lungs (freshly obtained from the slaughter-house) was extracted with 600 cc. of ice-cold physiological saline for 3 hours. The mixture was pressed out through a canvas bag and the filtrate (375 cc.) was subjected to three centrifugations of 45 minutes each in a refrigerated angle centrifuge at 4200 R.P.M. The turbid, reddish supernatant (pH 6.4) served for the isolation of the thromboplastic protein in the ultracentrifuge. This operation was carried

³ We wish to thank Dr. John Lee of Hoffmann-La Roche, Inc., Nutley, New Jersey, for this preparation.

⁴ We are very grateful to Dr. M. Bergmann of The Rockefeller Institute for Medical Research, for a specimen of this compound.

out in an air-driven vacuum ultracentrifuge (25) holding ten tubes, each of a capacity of 6 cc. Before each centrifugation the rotor was chilled to 4°; since it was operated in a vacuum chamber, no great increase in temperature took place during a run.

In each experiment between 60 and 180 cc. of the lung extract, clarified in the angle centrifuge, were used. The solutions were centrifuged at 42,000 R.P.M. for 25 minutes, and the resulting pellets dissolved in about one-third of the original volume of borate buffer⁵ of pH 8.6 and again sedimented at 42,000 R.P.M. The sediments, dissolved in the same amount of buffer as before, were subjected to centrifugation at 7500 R.P.M. for 20 minutes, in order to remove undissolved particles, and the material was alternately centrifuged at high and low speeds three to five times. Following the third sedimentation at 42,000 R.P.M., the supernatants became completely clear and colorless. Finally, the white pellets were dissolved in borate buffer of pH 8.6 and the colorless turbid solutions used for the physical measurements described in the following section. These preparations, obtained in individual runs, are designated with roman numerals.

The sediments collected in the last two low speed centrifugations (7500 R.P.M.), representing material sedimented in the ultracentrifuge which could not be brought back into stable solution, were likewise examined. They were taken up in borate buffer of pH 8.6, the solution was centrifuged at 4200 R.P.M., and the supernatant dialyzed against running tap water for 48 hours and against frequent changes of ice-cold distilled water for the same period of time. Concentration in a high vacuum of the solutions in the frozen state led to the isolation of the dried protein preparations (designated by the roman numeral corresponding to the experiment followed by 1, e.g. Preparation I-1). Similarly, the remainders from the highly purified final solutions which served in the experiments to be described in the following sections were in several cases dialyzed and concentrated in the frozen state; they are designated Preparations I-2, II-2, etc. The solid preparations formed an extremely light and voluminous, hygroscopic, white felt. The yield of the product in its final stage of purification may be estimated as 65 mg. per 100 gm. of tissue. Data on the composition of a number of such preparations will be found in Table II.

Physical Properties of Thromboplastic Protein

Ultracentrifuge Studies—The sedimentation velocity was determined by means of an analytical rotor (thickness of cell 0.5 cm., capacity 0.4 cc.). The course of the sedimentation was observed by the sehlieren method in the arrangement described by Chiles and Severinghaus (26). Because of

⁵ In all experiments borate buffers (ionic strength 0.15) were employed.

the high weight of the protein, the centrifugations were carried out at 7500 R.P.M. The speed of the centrifuge was measured by means of a Strobotac, constructed by the General Radio Company. The average temperature was 27°. The boundaries observed were sharp; a single component only was detected in all experiments. Within certain limits the sedimentation constants were not influenced by the concentration of the protein. At a high concentration slower sedimentation was observed in one case (Experiment 1, Table I).

Diffusion—The diffusion constants were determined in the Tiselius apparatus at 1.5°; an infra-red-sensitive plate (27) was used. The protein solutions in borate buffer were dialyzed in the refrigerator against a large volume of the same buffer. The diffusion constants were calculated according to Lamm and Polson (28).

Partial Specific Volume—The densities of the buffer and the protein solution were determined at 27° by means of the falling drop method.⁶ A borate buffer of pH 8.5 (ionic strength 0.15) was used. In order to obtain adequate falling times, *o*-fluorotoluene containing a very small amount of bromobenzene was employed as immiscible liquid. The falling times of the unknown solutions were compared with sodium chloride solutions of known density. The density of the borate buffer was found to be $d_4^{27} = 1.00284$, that of the protein solution (0.803 per cent concentration), $d_4^{27} = 1.00385$. The partial specific volume of the protein, therefore, was $V_{27} = 0.87$.

Viscosity—The specific viscosity of a 0.43 per cent solution of the protein (Preparation IV) was 1.06 at 30.6°.

Electrophoretic Mobility—The electrophoretic properties of Preparation I were examined at pH 8.3, those of Preparation IV at pH 7.5 and 8.6 (Table I, Experiments 1, 7, 8). The electrophoresis runs were carried out in the Tiselius apparatus at 1.5°, with the optical arrangement of Longworth (30) and an infra-red-sensitive plate (27). Only one component was observed with the mobilities (calculated on the basis of the descending boundaries) given in Table I.

Thromboplastic Activity

The clotting tests were carried out with rooster plasma in the customary arrangement (9). Each tube contained 0.1 cc. of plasma and 0.03 cc. of the solution of the protein in borate buffer of pH 8.3. The temperature

⁶ We are highly indebted to Dr. D. Rittenberg of this Department for help and advice with regard to these measurements. The experimental arrangement has been described (29).

was 30.5°. A typical experiment with a very active preparation (No. II-2) is reproduced, as follows

	Protein in experiment, γ					
	0.67	0.22	0.074	0.024	0.008	0
Clotting time, min..	3	5	8	11	17	30

It appeared of interest to test the homogeneity of the preparations, as shown in ultracentrifuge and electrophoresis experiments, in a different way. A specimen of Preparation IV (Table I) was subjected to prolonged electrophoresis, at pH 8.6. The contents of the electrophoresis cell then were arbitrarily divided into three portions, designated "fast," "middle," and "slow" fractions. (For the experimental arrangement, compare (31).

TABLE III
Thromboplastic Activity of Preparation IV after Electrophoresis

	Protein nitrogen in experiment			
	1.14 γ	0.38 γ	0.13 γ	0
	Clotting time			
	min	min.	min	min
Fast fraction.....	4	7	17	>60
Middle ".....	4	7	11	>60
Slow ".....	5	8	11	>60

In the present experiments, the "fast" fraction consisted of a small portion taken from the upper edge of the ascending boundary, the "middle" fraction of a portion taken from the middle section of the cell, the "slow" fraction of a small portion removed from the upper edge of the descending boundary.) The solutions were adjusted to the same nitrogen concentration and tested for thromboplastic activity. The results, reproduced in Table III, revealed no appreciable difference in the activities of the three fractions. It may be mentioned that the thromboplastic protein preparations failed to clot purified fibrinogen.

Phosphatase Activity

Splitting of Sodium β -Glycerophosphate—The decomposition of β -glycerophosphoric acid by preparations of the thromboplastic protein was followed by a method essentially similar to that of Albers (32, 33). In each experiment, 15 cc. of a 0.2 per cent solution of crystalline sodium β -glycerophosphate (containing a total of 2.95 mg. of P) were mixed with 5 cc. of veronal

buffer of pH 9.1 and a solution of 2 to 4 mg. of the thromboplastic protein. The mixture, in some cases after the addition of 0.02 mm of magnesium sulfate or calcium chloride, was made up to 25 cc. with water and kept at 35°. The inorganic phosphorus content was determined colorimetrically in aliquots removed at the beginning of the experiments and after 15, 30, and 60 minutes. The samples examined at 0 time contained no inorganic phosphorus. The action of a number of preparations of the thromboplastic protein is reproduced in Fig. 1. It will be seen that the addition of Mg^{++} enhanced the enzymatic activity, whereas Ca^{++} depressed it.

The phosphatase activities of the various preparations may be compared by computing the phosphatase units per mg. (33). 1 phosphatase unit is

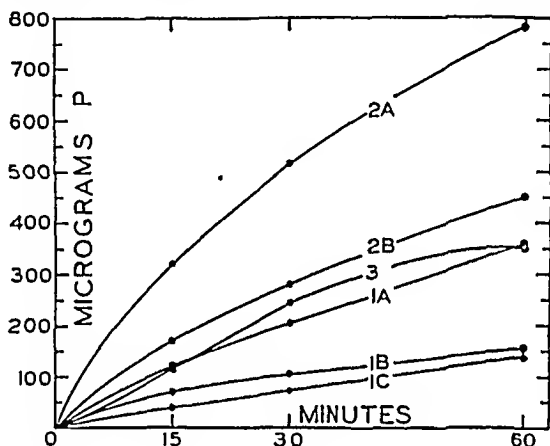


FIG. 1. Dephosphorylation of sodium β -glycerophosphate. Curve 1, Preparation IV; A with $MgSO_4$, B without addition, C with $CaCl_2$. Curve 2, Preparation II-2; A with $MgSO_4$, B without addition. Curve 3, Preparation IV-1, with $MgSO_4$.

defined as the amount of enzyme which under optimal conditions liberates 100 γ of inorganic P from β -glycerophosphoric acid in 1 hour. Preparation II-2 thus was found to contain 2.6, Preparation IV 1.7, and Preparation IV-1 0.9 phosphatase units per 1 mg. of protein.

If it is desired to compare the initial activities of the enzyme, one can, by a procedure similar to that of Bodansky (34), use an expression containing the time required for the liberation of 100 γ of inorganic P under the conditions of the experiment. In the equation $A_{100} = 100/tc$, A_{100} is the initial activity of 1 mg. of the enzyme preparation and t the time in minutes (determined graphically) required for the formation of 100 γ of inorganic P by c mg. of the enzyme. For Preparations II-2, IV, and IV-1, A_{100} (in the presence of Mg^{++}) was found to be 7.02, 3.96, and 1.92 respectively.

It may be pointed out that the error introduced by the eventual decomposition of the protein itself was not to be feared, since the contribution of the protein could, under the most adverse conditions (*viz.* complete hydrolysis), have amounted to no more than 50 γ of phosphorus.

Splitting of Sodium 2-Methyl-1,4-naphthohydroquinone Diphosphate—In each experiment 22.2 mg. of the tetrasodium salt (35, 36), corresponding to a total of 3.0 mg. of P, were dissolved in 5 cc. of veronal buffer of pH 9.1, 4.0 mg. of Preparation IV-1 were added, and the mixtures, in some cases after the addition of 0.02 mM of magnesium sulfate or calcium chloride, diluted with water to a volume of 25 cc. The experiments were carried out at 35°. The results, shown in Table IV, were corrected for the appreciable spontaneous decomposition of the phosphoric acid ester: 22.2 mg. of the sodium salt gave rise to 107 γ of inorganic P after 30 minutes and to 125 γ after 1 hour at pH 9.1 and 35°. No inorganic P could be detected at

TABLE IV

Dephosphorylation of Sodium 2-Methyl-1,4-naphthohydroquinone Diphosphate by Preparation IV-1

	Inorganic phosphorus liberated in			
	0 min	15 min	30 min	60 min
	γ	γ	γ	γ
Without addition.	0	69	98	110
With MgSO_4	0	76	165	342
" CaCl_2	0	20	34	106

the start of the experiments. The enzymatic activity of this protein preparation, expressed in phosphatase units, was, as was to be expected, independent of the substrate used: 0.86 phosphatase unit per mg., compared with 0.9 unit with glycerophosphate. The initial activity was somewhat lower: A_{100} (in the presence of Mg^{++}) was 1.35 with methylnaphthohydroquinone phosphate, 1.92 with glycerophosphate.

Examination for Trypsin Action

In these experiments α -benzoyl-L-argininamide hydrochloride (37) was used as substrate. In each experiment between 2 and 3 mg. of the protein preparations (Preparations II-2 and IV-1) and 0.045 mM of the substrate dissolved in M/15 phosphate or borate buffers of pH 7.8 were used. The total volume in each experiment was 1 cc., the temperature 40°. The reaction was followed titrimetrically according to the method of Grassmann and Heyde (38). The titrations were carried out by means of an accurate micrometric burette (39) and an electrical stirring arrangement. The

results were inconclusive, since the protein preparations themselves were unstable under the experimental conditions and slowly gave rise to basic substances, thereby obscuring an eventual enzymatic action. In control experiments with Fairchild trypsin, definite splitting of the synthetic substrate occurred. After 1 hour 28 per cent and after 3 hours 57 per cent of the substrate were decomposed.

SUMMARY

The fractional ultracentrifugation of saline extracts of beef lung led to the isolation of a high molecular lipoprotein with a very high thromboplastic activity. This fraction was homogeneous both in the electrophoresis cell and in the ultracentrifuge. A particle weight of 167×10^6 was calculated from the rate of sedimentation ($s_{20} = 330$ S), diffusion measurements ($D_{20} = 0.38 \times 10^{-7}$), and partial specific volume ($V_{27} = 0.87$). Electron micrographs revealed the presence of spherical particles with a diameter of 80 to 120 m μ .

The thromboplastic activity of as little as 0.008 γ of the protein could still be demonstrated by clotting tests. The thromboplastic protein preparations also exhibited marked phosphatase activity towards sodium β -glycerophosphate and sodium 2-methyl-1,4-naphthohydroquinone diphosphate, a compound having high vitamin K activity.

The nature of the thromboplastic effect is discussed in the light of these findings.

BIBLIOGRAPHY

1. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **140**, 689 (1941).
2. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **136**, 243 (1940).
3. Chargaff, E., Ziff, M., and Cohen, S. S., *J. Biol. Chem.*, **136**, 257 (1940).
4. Cohen, S. S., and Chargaff, E., *J. Biol. Chem.*, **139**, 741 (1941).
5. Svedberg, T., and Pedersen, K. O., *The ultracentrifuge*, Oxford, 38 (1940).
6. Polson, A., *Kolloid-Z.*, **88**, 51 (1939).
7. Simha, R., *J. Physic. Chem.*, **44**, 25 (1940).
8. Mehl, J. W., Oneley, J. L., and Simha, R., *Science*, **92**, 132 (1940).
9. Chargaff, E., Bancroft, F. W., and Stanley-Brown, M., *J. Biol. Chem.*, **116**, 237 (1936).
10. Folch, J., and Schneider, H. A., *J. Biol. Chem.*, **137**, 51 (1941).
11. Milstone, H., *J. Gen. Physiol.*, **25**, 679 (1942).
12. Bordet, J., *Ann. Inst. Pasteur*, **34**, 561 (1920).
13. Chargaff, E., Ziff, M., and Cohen, S. S., *J. Biol. Chem.*, **135**, 351 (1940).
14. Howell, W. H., *Physiol. Rev.*, **15**, 435 (1935).
15. Morawitz, P., in Oppenheimer, C., *Handbuch der Biochemie*, Jena, **4**, 44 (1925).
16. Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, **20**, 543 (1937).
17. Heard, W. N., *J. Physiol.*, **51**, 294 (1917).
18. Eagle, H., *J. Exp. Med.*, **65**, 613 (1937).
19. Lee, J., Solmsen, U. V., Steycrmark, A., and Foster, R. H. K., *Proc. Soc. Exp. Biol. and Med.*, **45**, 407 (1940).

20. Stern, K. G., and Duran-Reynals, F., *Science*, **89**, 609 (1939).
21. Claude, A., *Science*, **91**, 77 (1940).
22. Henle, W., and Chambers, L. A., *Science*, **92**, 313 (1940).
23. Kabat, E. A., *Science*, **93**, 43 (1941).
24. Gard, S., and Pedersen, K. O., *Science*, **94**, 493 (1941).
25. Chiles, J. A., Jr., and Severinghaus, A. E., *J. Exp. Med.*, **68**, 1 (1938).
26. Chiles, J. A., Jr., and Severinghaus, A. E., *Rev. Scient. Instruments*, **11**, 71 (1940).
27. Treffers, H. P., and Moore, D. H., *Science*, **93**, 240 (1941).
28. Lamm, O., and Polson, A., *Biochem. J.*, **30**, 528 (1936).
29. Keston, A. S., Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **122**, 227 (1937-38).
30. Longworth, L. G., *J. Am. Chem. Soc.*, **61**, 529 (1939).
31. Chargaff, E., Ziff, M., and Moore, D. H., *J. Biol. Chem.*, **139**, 383 (1941).
32. Albers, H., and Albers, E., *Z. physiol. Chem.*, **232**, 165 (1935).
33. Albers, H., in Nord, F. F., and Weidenhagen, R., *Handbuch der Enzymologie*, Leipzig, 408 (1940).
34. Bodansky, O., *J. Biol. Chem.*, **118**, 341 (1937).
35. Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, **62**, 228 (1940).
36. Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, **62**, 453 (1940).
37. Bergmann, M., Fruton, J. S., and Pollok, H., *J. Biol. Chem.*, **127**, 643 (1939).
38. Grassmann, W., and Heyde, W., *Z. physiol. Chem.*, **183**, 32 (1929).
39. Scholander, P. F., *Science*, **95**, 177 (1942).

THE HIGHER FATTY ALDEHYDES

I. ISOLATION FROM SMALL AMOUNTS OF TISSUE WITH ACIDIC CARBONYL REAGENTS*

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In this paper the isolation of the higher fatty aldehydes from relatively small amounts of beef muscle, beef brain, rat muscle, and rat brain is reported, as a first essential step in the study of their metabolic activity. At the present time nothing is known about the function of these aldehydes, which were first isolated from biological material by Feulgen and collaborators (2, 3). A possible indication of high metabolic activity of these compounds was obtained from the results of a fractionation of the unsaponifiable material of rats kept on a heavy water régime (4). The concentration of deuterium in the alcoholic fraction not precipitated by digitonin was twice as high as that in the cholesterol fraction or in the ketonic fraction. It was suggested that the alcoholic fraction may have been derived from the higher fatty aldehydes, to which a high metabolic activity might therefore be ascribed. Such a relationship is supported by the isolation of batyl alcohol from vertebrates (5). It has already been suggested (6) that this glycerol ether may have its origin in the acetal phosphatides which, according to Feulgen, contain the aldehydes in acetal linkage.

The aldehydes are liberated by the action of mercuric chloride or acid in an aqueous emulsion of the tissue lipids (2). Feulgen and collaborators isolated the thiosemicarbazone of an aldehyde mixture from horse muscle, using steam distillation for the separation of the aldehydes from other lipids (1.5 gm. of thiosemicarbazone from 10 kilos of horse muscle). According to him the "plasmal" fraction isolated as the less alcohol-soluble thiosemicarbazone represents a mixture of aldehydes having a chain length of C_{16} and C_{18} , the thiosemicarbazone of at least one other aldehyde remaining in the mother liquor. He did not succeed in isolating from brain tissue a thiosemicarbazone corresponding to that obtained from muscle, and concluded that the aldehydes from brain represent a mixture of a different ratio. Behrens (3) isolated the thiosemicarbazone without steam

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distillation of the aldehydes, taking advantage of the insolubility of the silver derivative of the thiosemicarbazone. This method did not improve the yields obtained with Feulgen's original procedure, and was not applicable to brain.

We have utilized the carbonyl reagents *p*-carboxyphenylhydrazine and carboxymethoxylamine for the isolation of the higher fatty aldehydes. The derivatives can be separated from the lipid mixture on the basis of their acidic properties. Our methods differ from those of Feulgen and of Behrens in respect to the aldehyde derivative isolated and in the procedure used to obtain the lipid emulsion. For muscle an acetone extract was used; for brain, an ether extract of the acetone-dried tissue. In both cases hot alcohol extraction was used as an alternate procedure. The aldehydes were liberated in the emulsion by treatment with acid. They were separated from the bulk of lipids by acetone precipitation. Coupling with the carbonyl reagents and isolation of the derivatives were carried out essentially according to Anchel and Schoenheimer (7).

The concentration of aldehydes actually present in the tissue was measured by a modification of the colorimetric procedure of Feulgen and Gruenberg (8, 9).

We have succeeded in obtaining the free aldehydes by splitting the derivatives with pyruvic acid. The aldehydes thus liberated can be converted into other aldehyde derivatives.

By treating the acid hydrazones or oximes with an excess of a neutral aldehyde reagent, a different aldehyde derivative is obtained without exposing the aldehydes to the danger of polymerization, since they are transferred directly from one reagent to another. The new derivative which is neutral can be separated from the original acid aldehyde reagent.

EXPERIMENTAL

The methods of isolation of aldehyde derivatives are described for 200 gm. of muscle and brain tissue but have been used for amounts up to 5 kilos.

Preparation of Lipid Emulsion—With beef muscle two methods of obtaining the emulsion have proved reliable. (a) 200 gm. of ground muscle were extracted three times for 20 minute periods with 100 ml. of boiling ethanol. The alcoholic filtrates were combined and concentrated *in vacuo* and the residue was emulsified in 70 ml. of water. (b) 200 gm. of ground muscle were treated with 1600 ml. of acetone at room temperature with occasional stirring for 1 hour. The main portion of the acetal phosphatides was found in the first acetone extract, the second giving a color test corresponding to not more than 5 per cent of the amount of aldehyde indicated in the first extract. The acetone was removed *in vacuo* and the residue was emulsified in 70 ml. of water.

The first emulsions from beef brain were obtained with the hot alcohol procedure, but in the procedure finally adopted a dry tissue powder was prepared from brain by chopping the tissue in a mechanical mixer and treating it for three 1 hour periods with 1600 ml. portions of acetone. The dry powder was extracted five times with 200 ml. of boiling ether and the residue of the combined ether extracts emulsified in 100 ml. of water.

The coagulation of the tissue with hot water and the alcohol extraction at room temperature as proposed by Feulgen have no advantage over our extraction procedures, which can be completed in a few hours. For consistent isolation of the aldehyde derivatives it is necessary to remove a large part of the lipids present despite the fact that this entails a loss of acetal phosphatides by adsorption. The alcohol-insoluble lipids are filtered off after the alcoholic extracts have been kept for about 10 hours. If the precipitate shows a strong fuchsin test, it is recrystallized from ethanol and the mother liquor combined with the first alcoholic extract. The same result can be attained more simply by discarding the first alcohol extract, which usually gives a very weak fuchsin test.

Liberation of Aldehydes by Acid Treatment of Lipid Emulsion—The emulsion was acidified with dilute sulfuric acid to pH 1 to 2. Varying amounts of acid were necessary because of the different buffering capacity of the emulsion. Depending on the extent of removal of alcohol-insoluble lipids, 1 to 4 ml. of 2 N sulfuric acid was needed for 100 ml. of emulsion. The acidified emulsion from beef muscle was stirred for 4 hours at 37° or up to 16 hours at room temperature. Emulsion from brain needs longer acid treatment than that from muscle (8 to 16 hours at 37°).

Preparation of Aldehydes for Reaction with Carbonyl Reagents—100 ml. of the acid-treated emulsion were distributed between 150 ml. of petroleum ether and 150 ml. of a 4 per cent solution of potassium carbonate to remove all acidic products (e.g. fatty acids) which might have been formed during the acid treatment. For clearing the resulting emulsion 150 ml. of ethanol were added. The carbonate extraction was repeated three times and the petroleum ether extract was washed neutral with water. Acidification of the carbonate extracts showed that the degree of hydrolysis to fatty acids is very small. The petroleum ether extract was concentrated *in vacuo* and the residue was taken up in a sufficient amount of ether. To remove additional lipids the ethereal solution was poured into 7 volumes of acetone. At this step some aldehyde was lost. This loss is minimized if the solution is fairly dry and is poured into boiling acetone with stirring. The acetone filtrate was concentrated *in vacuo* and taken to dryness twice with addition of alcohol. The residue, corresponding to 200 gm. of tissue, was dissolved in 20 ml. of ethanol.

Coupling Aldehydes with Carbonyl Reagents—The alcoholic solution of

free aldehydes was refluxed with the carbonyl reagents for 2 hours (0.25 gm. of *p*-carboxyphenylhydrazine, Eastman, or of carboxymethoxylamine (10) per 200 gm. of beef muscle or brain). The *p*-carboxyphenylhydrazine if partly oxidized, as indicated by a brown color, should be purified (7). With the *p*-carboxyphenylhydrazine glacial acetic acid was added (0.5 ml. per gm. of reagent) and with the carboxymethoxylamine anhydrous sodium acetate (0.75 gm. per gm. of reagent).

After the reaction the alcoholic solution was poured into 4 volumes of a 4 per cent solution of potassium carbonate (1 per cent in the case of the oximes). The mixture was extracted with 4 volumes of ether. After the emulsion had cleared, the ether was washed with carbonate solution until no further precipitate appeared on acidification of small aliquots. The combined extracts were acidified with hydrochloric acid. A solid precipitate was best obtained by cooling the carbonate solution before acidification and removing the dissolved ether by a stream of inert gas. The hydrazones were filtered under a stream of inert gas or were centrifuged.

The behavior of the *p*-carboxyphenylhydrazones on distribution depends on the relative amount of accompanying lipids and on the amount of alcohol used. It sometimes happens that hydrazones are held back in the ether from which they can be extracted by varying the amounts of the distributing solvents. An ether layer more deeply colored than the carbonate solution is usually an indication of such a situation. Sometimes a middle layer appears which probably consists of a mixture of hydrazones and lipids. If this layer forms a clear solution with carbonate, it can be acidified directly. Otherwise a redistribution between ether and carbonate is advisable.

The *p*-carboxyphenylhydrazones were recrystallized from aqueous ethanol. From glacial acetic acid better crystals were obtained but in smaller yields. The carboxymethoximes were recrystallized from methanol.

Since we are dealing presumably with a mixture of aldehydes, the analytical data can serve only for identification. The *p*-carboxyphenylhydrazones vary in color from a light cream to a deep brown, depending on the state of purity and exposure to oxidation. The carboxymethoximes are white.

The *p*-carboxyphenylhydrazones from beef muscle and beef brain from different sources melted at 95–100°, with softening from 80–85°. The melting point of the carboxymethoximes was between 67–72°. For analysis the hydrazones and oximes were recrystallized twice and dried over phosphorus pentoxide at 2 mm. and room temperature.¹

¹ We are indebted to Mr. W. Saschek for the analyses. The nitrogen values of the hydrazones as determined with the micro-Dumas procedure are too low, an observation often encountered with compounds of this type.

p-Carboxyphenylhydrazones from Beef Brain (Fig. 1) and Beef Muscle—Found, C 73.72 to 74.36, H 10.37 to 10.57, N 6.46 to 6.92; calculated for $C_{24}H_{40}O_2N_2$ (388.6), C 74.18, H 10.38, N 7.21.

Carboxymethoximes from Beef Brain and Beef Muscle (Fig. 2)—Found, C 69.24 to 69.59, H 11.33 to 11.44, N 3.98 to 4.01; calculated for $C_{19}H_{37}O_3N$ (327.4), C 69.67, H 11.38, N 4.27.

For identification of the aldehyde derivatives in routine isolations, the titration of the carboxyl group in alcoholic solution was employed. The



FIG. 1. *p*-Carboxyphenylhydrazones of higher fatty aldehydes from beef brain



FIG. 2. Carboxymethoximes of higher fatty aldehydes from beef muscle

values found agree within ± 5 per cent with a molecular weight calculated for an aldehyde mixture with an average chain length of C_{17} .

The yields of aldehydes obtained as crude *p*-carboxyphenylhydrazones or carboxymethoximes varied between 0.05 and 0.2 per cent of the weight of beef muscle used. For beef brain the yields corresponded to 0.1 to 0.2 per cent. The yields after one recrystallization, which gave products with good analytical composition, amounted to one-third to one-half of these

values. Feulgen obtained 1.5 gm. of thiosemicarbazones from 10 kilos of horse muscle, a yield of 0.015 per cent. Our yields of crude aldehyde derivative from beef muscle appear to approach the amounts determined colorimetrically, whereas the yields from brain tissue are far lower.

In one experiment three 200 gm. samples of chopped beef muscle were extracted with acetone. The colorimetric procedure indicated 209, 210, and 213 mg. \pm 10 mg. of aldehyde in the first acetone extract. The second acetone extract contained color-producing substance amounting to not more than 1 per cent of that of the first. As a standard, free aldehydes obtained by splitting the hydrazones were used. The first recrystallization yielded hydrazones corresponding to 61, 82, and 66 mg. of free aldehyde. The mother liquor contained residues corresponding to 120, 60, and 50 mg. respectively of free aldehyde. In this particular experiment about 30 per cent of the aldehydes was isolated in pure form. The residue in the mother liquor corresponded to another 25 to 60 per cent.

Isolation of Aldehyde Derivates from Rat Tissue—Considerable difficulties were experienced in the isolation of the *p*-carboxyphenylhydrazones from rat tissue. We have succeeded in isolating the aldehydes from sets of adult and new born rats in several experiments but a great number of negative experiments have shown that much more has to be known about the biological factors affecting the concentration of aldehydes in rat tissue before a reliable routine procedure for their isolation can be developed.

The following experiments demonstrate that the aldehyde derivatives isolated from rat tissue are very similar in properties to those isolated from beef muscle and beef brain. With the hot alcohol procedure 900 gm. of ground muscle and bone from five adult rats gave 0.43 gm. of once recrystallized *p*-carboxyphenylhydrazones melting at 92°. Found, C 73.69, H 10.37. By the same procedure 450 gm. of ground muscle and bone from adult rats yielded 0.15 gm. of crude carboxymethoximes and 40 mg. of recrystallized material melting at 65°. Found, C 70.02, H 11.5 (0.3 per cent ash). 200 gm. of ground carcasses (including viscera but without heads or skins) of young rats up to the age of 10 days gave 0.1 gm. of crude hydrazones melting at 95°. From 10 gm. of brain (first rat experiment) 87 mg. of crude *p*-carboxyphenylhydrazones were isolated. Recrystallized with great loss, it melted at 95°. Found, C 72.43, H 10.38.

Direct Transfer of Aldehydes from One Reagent to Another—0.1 gm. of *p*-carboxyphenylhydrazones from beef muscle was refluxed in 10 ml. of 95 per cent acetic acid with 0.5 gm. of thiosemicarbazide (20 moles) for 8 hours. After distribution between ether and carbonate the ethereal solution contained 71 mg. of neutral residue, which after recrystallization from ethanol and ethyl acetate gave 22 mg. of thiosemicarbazones melting at 100–102°. Found, S 9.57; calculated for $C_{18}H_{36}N_3S$ (326.59), S 9.82.

Splitting p-Carboxyphenylhydrazones from Beef Muscle—90 mg. of hydrazones were refluxed with 200 mg. of redistilled pyruvic acid in 10 ml. of 95 per cent ethanol for 10 hours. The alcoholic solution was distributed between ether and 4 per cent potassium carbonate solution. After acidification the carbonate solution yielded 44 mg. of pyruvic acid hydrazone melting at 225° (90 per cent of theory). The washed and dried ether contained a residue of 60 mg. (100 per cent of theory). 54 mg. were allowed to react again with *p*-carboxyphenylhydrazine and yielded 45 mg. of hydrazones and 13 mg. of unreactive material which gave a fuchsin test corresponding to about 1 mg. It is assumed that this unreactive part of the residue represents polymerized aldehydes. On this assumption 80 per cent of the aldehydes has been accounted for.

The carboxymethoximes can be split with pyruvic acid in a similar way. Splitting with hydrochloric acid leads to an ether residue which gives only a faint fuchsin test and which cannot be recombined to form derivatives. It consists presumably of polymerized aldehydes.

DISCUSSION

The aldehydes isolated from beef muscle, beef brain, rat muscle, and rat brain are very similar in nature, judging from the analytical values of the derivatives. These values indicate the presence of aldehydes of an average chain length of C₁₆ to C₁₈, a finding which supports the belief that the aldehydes consist mainly of stearaldehyde and palmitaldehyde. It cannot be assumed that these aldehydes are the only ones present, since the crystallized and analyzed aldehyde derivatives represent only about 30 per cent of the crude precipitate of aldehyde derivatives obtained. The mother liquor may contain a variety of other aldehydes. It must be kept in mind that particularly in the case of brain only a small part of the aldehyde present has been isolated, if the colorimetric test is to be taken as an indication of its actual concentration.

Since the aldehydes are isolated directly from the lipid fraction, derivatives of other carbonyl compounds may be present; *e.g.*, ketosteroids. The concentration of these is probably too small to interfere seriously with the analytical results.

The isolation of the higher fatty aldehydes from small amounts of tissue with the aid of the acid carbonyl reagents has made possible the study of their metabolic function in small laboratory animals. The colorimetric test indicates large variations of the aldehyde concentration in the muscle of different animals, as has been noticed also in histological studies (11). The ratio of fuchsin-positive substance in beef to that in rat muscle is sometimes as high as 5:1. This may be one of the reasons for the difficulties experienced in the isolation of the aldehydes from rat muscle.

Furthermore, the relative ratio of the aldehyde mother substance to other lipids appears to influence the success of the isolation procedure. The importance of these two factors is clearly shown by the different behavior of beef muscle and beef brain towards acetone. Practically all of the aldehyde mother substance can be removed from beef muscle by one acetone extraction, but only a small fraction from beef brain.

The free aldehydes are easily obtained by splitting the *p*-carboxyphenylhydrazones or carboxymethoximes with pyruvic acid. This behavior appears to be unlike that of the Girard aldehyde compounds which in general cannot be readily split (12). The fact that it was not possible to recombine all of the liberated aldehyde may indicate polymerization, since the coupling reaction itself is probably quantitative. The use of mineral acid instead of pyruvic acid leads to complete polymerization, as indicated by the negative fuchsin test in the ether-soluble fraction.

Other aldehyde derivatives can be prepared without isolating the free aldehyde, by treatment of one of the derivatives with an excess of another carbonyl reagent. This type of transfer reaction may be useful when dealing with other labile carbonyl compounds.

SUMMARY

Methods are described for the isolation of the higher fatty aldehydes as *p*-carboxyphenylhydrazones and carboxymethoximes from beef muscle, beef brain, rat muscle, and rat brain. The isolation procedure has been applied successfully to small amounts of tissue (200 gm. of beef muscle, 10 gm. of rat brain). The yields of the crude aldehyde derivatives amount to 0.05 to 0.2 per cent of the tissue used.

The composition of the top fraction of the recrystallized aldehyde derivatives from different sources corresponds to the presence of aldehydes of an average chain length of C_{16} to C_{18} .

Free aldehydes were obtained by splitting the derivatives with pyruvic acid.

A method for the direct transfer of the aldehydes from one carbonyl reagent to another is described.

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BIBLIOGRAPHY

1. Waelsch, H., and Anchel, M., *Federation Proc.*, **1**, pt. 2, 139 (1942).
2. Feulgen, R., and Voit, K., *Arch. ges. Physiol.*, **206**, 390 (1924). Feulgen, R., Imhauser, K., and Behrens, M., *Z. physiol. Chem.*, **180**, 161 (1929). Feulgen, R., and Behrens, M., *Z. physiol. Chem.*, **256**, 15 (1938). Feulgen, R., and Bersin, T., *Z. physiol. Chem.*, **260**, 217 (1939).

3. Behrens, M., *Z. physiol. Chcm.*, **191**, 183 (1930).
4. Waelsch, H., and Sperry, W. M., *J. Biol. Chem.*, **132**, 787 (1940).
5. Holmes, H. N., Corbét, R. E., Geiger, W. B., Kornblum, N., and Alexander, W., *J. Am. Chem. Soc.*, **63**, 2607 (1941).
6. Baer, E., and Fischer, H. O. L., *J. Biol. Chem.*, **140**, 397 (1941).
7. Anchel, M., and Schoenheimer, R., *J. Biol. Chem.*, **114**, 539 (1936).
8. Feulgen, R., and Gruenberg, H., *Z. physiol. Chem.*, **257**, 161 (1939).
9. Waelsch, H., *Proc. Am. Soc. Biol. Chem.*, *J. Biol. Chem.*, **140**, p. exxxvi (1941).
10. Borek, E., and Clarke, H. T., *J. Am. Chcm. Soc.*, **58**, 2020 (1936).
11. Verne, J., *Ann. physiol. et physicochim. biol.*, **5**, 245 (1929).
12. Girard, A., and Sandulesco, G., *Helv. chim. acta*, **19**, 1095 (1936).

THE RATE OF EXCRETION OF INGESTED ACID AND ALKALI

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The displacement of the acid-base balance of the body produced by the ingestion of acid- or alkali-forming substances is dependent on the buffering power of the body tissues and on the rate at which the acid or alkali is absorbed and excreted. After the ingestion of about 5 gm. of sodium bicarbonate, the alkali reserve and pH of the blood rise above the preingestion level but do not go beyond the normal range. After the ingestion of about 20 gm. of sodium bicarbonate, the alkali reserve and pH go beyond their normal ranges to reach maxima in about an hour and a half, and return to normal values within about 3 hours. Ammonium chloride produces a corresponding decrease in pH and alkali reserve but the maximum displacement occurs later and in some individuals the return to normal is more prolonged (1-3).

The early return of the alkali reserve of the blood to normal levels may be attributed to the passage of the alkali or acid into the tissues or into the urine. Accepted methods for the determination of total urinary acid or alkali (4, 5) are not well adapted to extended experimentation, either because they are too lengthy or because of uncertainty introduced in the organic acid titration by partial removal of citrate in preliminary urine treatment (6). It is the purpose of this paper to report a simple procedure for the determination of urinary alkali and the results obtained in its application to the determination of the rate of excretion of ingested acids and alkalies.

Methods

Urinary alkali was determined by subtracting urinary ammonia from urinary titratable alkalinity. Titratable alkalinity was determined by titrating a 30 cc. sample of urine with 0.25 N HCl to the pH of 0.002 N HCl (2.72), and correcting this titration by the amount of 0.002 N acid present in the final titration volume. 5 cc. of 0.015 per cent tropeolin OO solution were used as an indicator in the preliminary pH adjustment and the final adjustment was made with the aid of a glass electrode assembly. The solutions were shaken before aliquot portions were taken for analyses in order to suspend precipitated calcium phosphate evenly throughout the samples. Ammonia was determined by a modification of a procedure in which it is aerated into boric acid solution (7).

The theoretical basis for this procedure is found in the data appearing in Table I. Ingested alkali is excreted directly into the urine as bicarbonate, alkaline salts of phosphoric acid, and as salts of organic acids. The bicarbonate and alkaline salts of phosphoric acid are quantitatively determined in the alkali titration. The acid phosphate present originally in the urine and that arising from the titration of the alkaline salt are titrated to the extent of 13 per cent. Salts of citric acid are titrated to the extent of 87 per cent. Salts of other organic acids, such as lactic and β -hydroxybutyric, are excreted by the normal individual in negligible amounts and are almost quantitatively titrated. Urinary nitrogen compounds are titrated to

TABLE I
Titration of Urinary Alkali

Substance titrated	Products of reaction	Dissociation constant,* pK'	Amount† titrated at pH 2.72
			<i>per cent</i>
NaHCO_3	$\text{H}_2\text{CO}_3 + \text{NaCl}$	6.1	100
$\text{Ca}_1(\text{PO}_4)_2$	$\text{Ca}(\text{H}_2\text{PO}_4)_2 + \text{NaCl}$		100
Na_2HPO_4	$\text{NaH}_2\text{PO}_4 + \text{NaCl}$	6.6	100
NaH_2PO_4	$\text{H}_3\text{PO}_4 + \text{NaCl}$	2.0	13
Sodium citrate	Citric acid + 3NaCl		87
Creatinine	Creatinine hydrochloride	4.8	100
Urea	Urea hydrochloride	0.0	0.2
Glycine	Glycine hydrochloride	2.3	30
$(\text{NH}_4)_2\text{HPO}_4$	$(\text{NH}_4)_2\text{H}_2\text{PO}_4 + \text{NH}_4\text{Cl}$	6.6	100
$(\text{NH}_4)_2\text{H}_2\text{PO}_4$	$\text{H}_3\text{PO}_4 + \text{NH}_4\text{Cl}$	2.0	12

* The dissociation constants were taken from the literature (8-10).

† The amount titrated was calculated from the dissociation constant and determined experimentally. The values are in essential agreement with those of Van Slyke and Palmer (11). The value for the acid ammonium salt of phosphoric acid was determined from a solution of the sodium salt which contained an excess of ammonium chloride.

a varying degree. About 30 per cent of the amino acids, a very small fraction of the urea, and all the creatinine are included in the titration. Because of the constant diet these substances are excreted in constant amounts, and are titrated to the same extent in the control and experimental urine samples. Because ammonium is titrated to the same extent as the fixed base which it replaces in the urine, the amount was determined separately and subtracted from the titration of the total alkali. Electrometric titration curves of unidentified urine constituents indicate that important amounts of other substances which play a part in the acid-base balance are not excreted (8).

In summary of these considerations, it appears that under the conditions of our experiments the only sources of error in the determination of urinary alkali may be attributed to the failure to titrate all the increased urinary citrate and to the titration of 13 per cent of the acid phosphate. The error introduced by the failure to titrate all the citrate amounts to 13 per cent of the alkali excreted as citrate but to only 1 or 2 per cent of the total additional alkali excreted. The error introduced by the titration of 13 per cent of the acid phosphate is small and is dependent on the extent to which the ingestion of alkali causes phosphate to be excreted in the feces instead of the urine. In experimental work about 90 per cent of ingested alkali and acid was recovered in the urine. A large part of that which was not recovered in the urine was probably excreted by the intestine as insoluble calcium phosphate (12).

Results

Representative results obtained in the application of this procedure to the determination of the rate of excretion of ingested acid and alkali are shown in Table II. A diet of constant composition was ingested and urine samples were collected at intervals during the morning, the afternoon, and the evening of each day. Day 1 represents a normal control period. On Days 2 and 3, 10 gm. of sodium bicarbonate were ingested with breakfast just after the beginning of the collection of the first morning sample. The parallel rates at which alkali was excreted on these days and the prompt return of alkali excretion to the control level on Day 4 indicate that the alkali was quantitatively excreted within 24 hours after its ingestion. Amounts of ammonium chloride equivalent to the bicarbonate were ingested on Days 6, 7, and 8 of the experiment. During these successive days acid was excreted at an increasing rate, as indicated by the negative alkali excretion, the fastest rate of excretion occurring on each day about 4 hours after the ingestion of the ammonium chloride. Acid excretion continued for $2\frac{1}{2}$ days after the last ammonium chloride was ingested.

The ingestion of a more acid diet from Days 13 to 20 of the experiment is reflected in the excretion of smaller amounts of alkali during this period. On Days 17 and 18, when sodium chloride was ingested with breakfast, increased amounts of alkali were excreted. This was followed by a compensating decrease on Day 19. The increased alkali excretion caused by the ingestion of sodium chloride is probably compensatory to an increase in the ionic strength of the tissue fluids produced by the sodium chloride. The ingestion of sodium chloride decreases the alkali reserve of the blood (13), and the ingestion of sodium bicarbonate decreases blood chloride (14).

Table III summarizes the results of experiments on the rate of excretion of acid- and alkali-forming substances. The amounts of acid or alkaline

TABLE II

Urinary Excretion of Ingested Acid and Alkali (Subject A. K.)

Day No.	Substance ingested	Alkali excreted per hr.					Total excreted per day
		7.30-9.00 a.m.	9.00-10.30 n.m.	10.30 a.m.-12.00 m.	12.00 m.-5.00 p.m.	5.00 p.m.-7.30 a.m.	
		cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N	cc. 0.1 N
1*		25	10	9	5	28	503
2	NaHCO ₃ †	45	142	175	48	60	1636
3	"	76	218	179	61	49	1689
4		39	20	11	1	27	479
5		19	18	34	3	30	581
6	NH ₄ Cl‡	38	8	-15	-12	3	41
7	"	-3	-14	-21	-16	-10	-270
8	"	-17	-18	-28	-20	-15	-420
9		-21	-14	-17	-12	-5	-210
10		6	3	6	-3	26	406
11		26	41	37	13	27	607
12		31	23	15	1	35	569
13*							
14							
15		17	7	-3	20	10	267
16		25	1	3	25	5	245
17	NaCl§	0	29	10	6	19	380
18	"	4	20	1	14	14	310
19		5	-3	-6	-5	9	141
20		7	21	29	5	8	218

* The constant diet ingested during Days 1 to 12 of the experiment was more alkaline than the diet ingested from Days 13 to 20.

† 10 gm. of NaHCO₃ (1190 cc. 0.1 N) were ingested at 7.45 a.m.

‡ 6.4 gm. of NH₄Cl (1190 cc. 0.1 N) were ingested at 7.45 a.m.

§ 7.2 gm. of NaCl (1190 cc. 0.1 N) were ingested at 7.45 a.m.

TABLE III
Summary of Experiments

Substance ingested	No. of experiments	Subject	Per cent of ingested acid or alkali excreted*			
			After 2½ hrs.	After 4½ hrs.	After 9½ hrs.	After 24 hrs.
Sodium bicarbonate	23	†	23 ± 8			
	7	A. K.		44 ± 8	63 ± 9	90 ± 7
	1	J. K.		22	37	73
	1	"		24	64	101
" citrate	5	A. K.		40 ± 10	63 ± 8	88 ± 9
	1	J. K.		20	41	76
Calcium carbonate	3	A. K. }		3 ± 4	2 ± 3	-3 ± 5
	2	J. K. }				
Ammonium chloride	6	A. K.		9 ± 5	18 ± 6	59 ± 10
	1	J. K.		5	11	42

* The figures refer to the mean value and mean deviation. Two-thirds of the values falls within these limits.

† Performed on twenty-three different subjects.

substances administered were equivalent to 10 gm. of sodium bicarbonate. In the first group of experiments sodium bicarbonate was ingested by each of twenty-three individuals shortly after a breakfast which consisted of toast and coffee. The alkali excreted in $2\frac{1}{4}$ hours in excess of that excreted in control experiments amounted to 23 per cent of the ingested alkali. The remaining experiments were performed in the same way as the experiment shown in Table II. The alkali arising from sodium citrate was excreted at about the same rate as that coming from the bicarbonate, attesting to the very rapid rate at which citric acid is metabolized (15). No significant increase in alkali excretion followed the ingestion of calcium carbonate. The acid arising from ingested ammonium chloride was excreted at a slower rate than ingested alkali. Subject J. K. who had a history of renal damage during pregnancy about 6 months previously excreted both alkali and acid at a much slower rate than subject A. K.

SUMMARY

The alkali formed from ingested sodium citrate is excreted at approximately the same rate as the alkali of sodium bicarbonate. About 24 hours are required for complete elimination. Ingestion of calcium carbonate does not increase alkali excretion.

The acid arising from ingested ammonium chloride is excreted at a comparatively slower rate than ingested bicarbonate. About 2 days are required for complete elimination.

BIBLIOGRAPHY

1. Bischoff, F., Sansum, W. D., Long, M. L., and DeWarr, M. M., *J. Nutrition*, **7**, 51 (1934).
2. Cape, J., and Sevringhaus, E. L., *J. Biol. Chem.*, **121**, 549 (1937).
3. Shock, N. W., and Hastings, A. B., *J. Biol. Chem.*, **112**, 239 (1935-36).
4. Gamble, J. L., Ross, G. S., and Tisdall, F. F., *J. Biol. Chem.*, **57**, 633 (1923).
5. Albright, F., and Bauer, W., *J. Clin. Inv.*, **7**, 465 (1929).
6. Kuyper, A. C., *J. Biol. Chem.*, **123**, 405 (1938).
7. Sobel, A. E., Yuska, H., and Cohen, J., *J. Biol. Chem.*, **118**, 443 (1937).
8. Kuyper, A. C., *J. Biol. Chem.*, **123**, 409 (1938).
9. Clark, W. M., *The determination of hydrogen ions*, Baltimore, 3rd edition (1928).
10. Sendroy, J., Jr., and Hastings, A. B., *J. Biol. Chem.*, **71**, 783 (1926-27).
11. Van Slyke, D. D., and Palmer, W. W., *J. Biol. Chem.*, **41**, 567 (1920).
12. Zucker, T. F., *Proc. Soc. Exp. Biol. and Med.*, **18**, 272 (1921).
13. Ellis, A. W. M., *Quart. J. Med.*, **17**, 405 (1924).
14. Free, A. H., Davies, D. F., Gustafson, G. E., and Myers, V. C., *Federation Proc.*, **1**, pt. 2, 110 (1942).
15. Kuyper, A. C., and Mattill, H. A., *J. Biol. Chem.*, **103**, 51 (1933).

THE QUANTITATIVE ESTIMATION OF BOTH CYSTINE AND CYSTEINE IN MIXTURES

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Some years ago Sullivan (1-3) described a distinctive test for cystine—a reaction of cystine with sodium 1,2-naphthoquinone-4-sulfonate in a highly alkaline solution with the formation of a red color not discharged by reducing agents such as sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$). The test can be used for cystine (1-3) after reduction. In early work various means of reducing cystine to cysteine were tried: tin, or zinc, and hydrochloric acid, sodium amalgam, and sodium cyanide. Because of the simpler technique involved, treatment with 5 per cent aqueous sodium cyanide has been most frequently employed for the reduction of the disulfide bond.

The Sullivan reaction has been applied, in general, to the estimation of cystine, since cystine rarely occurs in hydrolysates, and when found therein, was converted to cysteine by air oxidation. When TiCl_3 was used to maintain reducing conditions and prevent humin formation (3, 4), the substance tested for was cysteine. Practically the same result was given whether the amino acid was estimated as cystine with cystine as the standard or as cysteine with cysteine as the standard.

However, in the study of viruses, hormones, and denatured proteins it became necessary to estimate cystine and cysteine in the presence of each other. To do this satisfactorily, it is necessary to understand the behavior of cystine and cysteine under the conditions of the Sullivan cyanide procedure.

In his first report on cystine, of which 2.0 mg. dissolved in 5.0 cc. of 0.1 N HCl were treated with 2.0 cc. of 5 per cent aqueous sodium cyanide and compared with 2.0 mg. of cysteine not treated with sodium cyanide, Sullivan (1) found practically 100 per cent conversion of cystine to cysteine by the sodium cyanide. The explanation of this finding is that enough naphthoquinone had been employed to satisfy the cystine requirements, but only one-half of that required for cysteine. With lower concentrations, cystine gave with the same quantities of reagents 50 to 75 per cent as much color as the same amount of cysteine. In 1929, Pulewka and Winzer (5) showed that cyanide reacts with cystine according to the equation, $\text{RSSR} + \text{KCN} = \text{RSK} + \text{RSCN}$.

* The data in this paper were taken in part from the dissertation presented by H. W. Howard in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Georgetown University.

In accordance with this reaction a mole of cystine should yield a mole of cysteine, and the chromogenic value of cystine would be 50 per cent that of an equal weight of cysteine. In recent work we have found that, mg. for mg., cystine treated with sodium cyanide gives one-half as much color as is given by chemically pure, anhydrous cysteine.¹ Variation from this relationship was found to be due to differences in the water content of the samples of cysteine hydrochloride and, in part, to oxidation.

Cysteine hydrochloride is hygroscopic. Shinohara (6) in an examination of three commercial samples found the water to be 6.37, 6.01, and 10.26 per cent and sums up his work with the statement, "Mere purification is not enough, as water is the principal impurity." In our work we have found that anhydrous cysteine hydrochloride takes up water rather readily and samples in use in the laboratory have contained from 9.6 to 15.7 per cent water. If kept in a vacuum desiccator over P_2O_5 , cysteine hydrochloride can be maintained free from moisture and, if dissolved in iron-free 0.1 N HCl, it is oxidized little in 24 hours.

The hypothesis that the action of cyanide on cystine is a double decomposition with the formation of 1 mole of cysteine and 1 mole of S-cyanocysteine was examined by comparison of the amount of cysteine formed from cystine by cyanide with the amount formed by complete reduction according to the equation $RSSR + H_2 = 2RSH$.

A suitable reducing agent for this purpose must reduce conveniently and completely in a reasonable time, without heating or filtration with the attendant risk of loss of cysteine by absorption, and without introducing into the solution material which would interfere with the subsequent color development. Reduction by sodium amalgam was found to meet these requirements.

Sodium Amalgam Reduction—The reagent employed was an approximately 0.2 per cent sodium amalgam prepared by dilution of a 2 per cent commercial preparation with mercury. The dilute amalgam possesses the advantage of being liquid at ordinary temperature and can be measured readily by volumetric methods. It reduces cystine to cysteine quantitatively at room temperature. 7 cc. of the cystine solution, 0.1 N with respect to HCl or H_2SO_4 and containing preferably 100 to 200 parts per million of cystine, are pipetted into a test-tube and 1.0 cc. of the amalgam is added. The mixture is allowed to stand for 1 hour with occasional shaking. Then 5.0 cc. of the reduced solution (a) are pipetted into a 25 by 2.5 cm. test-tube, and the cysteine content is determined in comparison with (b) the cysteine produced by the regular Sullivan procedure subsequently detailed.

¹ In all cases the cysteine was weighed out as the molar equivalent amount of cysteine hydrochloride.

Cystine was found to give twice as much color by the amalgam-cyanide method as by the cyanide-cystine method. Cysteine was found to give the same amount of color in both the amalgam-cyanide treatment and in the cyanide-cystine procedure. Treated by either method, cysteine gave the same color as an equal weight of cystine in the amalgam-cyanide procedure and twice as much color as an equal weight of cystine by the cyanide-cystine method. These relationships are summarized in Table I.

TABLE I
Comparison of Amalgam-Cyanide and Cyanide-Cystine Methods

Solution	Procedure	Colorimetric reading	Comparative chromogenic value
		mm.	per cent
200 p.p.m. cystine	Amalgam-cyanide	20	100 (Standard)
400 " "	Cyanide-cystine	20.5	49
200 " cysteine	Amalgam-cyanide	19.9	100
200 " "	Cyanide-cystine	19.3	103

TABLE II
Estimation of Cysteine and Cystine in Presence of Each Other

Amount present		Amount found		Total, cysteine + cystine
Cysteine	Cystine	Cysteine	Cystine	
mg.	mg.	mg.	mg.	mg.
0.1	0.9	0.095	0.908	1.003
0.2	0.8	0.209	0.817	1.026
0.3	0.7	0.319	0.695	1.014
0.4	0.6	0.409	0.609	1.018
0.6	0.4	0.591	0.411	1.002
0.7	0.3	0.675	0.321	0.996
0.8	0.2	0.779	0.213	0.992
0.9	0.1	0.911	0.105	1.016

A procedure for the quantitative estimation of cysteine and cystine in the presence of each other was developed from these findings. Two determinations are necessary: In Procedure 1 the determination of the chromogenic value by means of the regular Sullivan procedure with cystine as the standard is carried out in the following manner. To 5.0 cc. of solution containing 1.0 mg. of cystine, add 2.0 cc. of freshly prepared 5.0 per cent aqueous sodium cyanide, mix, and allow to stand 10 minutes at 20-25°; add 1.0 cc. of 1.0 per cent sodium 1,2-naphthoquinone-4-sulfonate and shake for 10 seconds; add 5.0 cc. of 10 per cent anhydrous sodium sulfite in 0.5 N NaOH, mix, and allow to stand 30 minutes. Then add 1.0 cc. of a 2 per

cent solution of sodium hyposulfite² in 0.5 N NaOH and match against a cystine standard similarly treated. In Procedure 2 reduce with sodium amalgam as described and treat as in Procedure 1 with cystine similarly reduced or c.p. cysteine hydrochloride as the standard. In solutions buffered against alkali, as in some protein hydrolysates, it may be necessary to add 1.0 cc. of 5 N NaOH before the hyposulfite.

The difference between the results of Procedures 1 and 2 gives the cystine present; this subtracted from the value found in Procedure 2 gives the cysteine present. An example is as follows: A mixture of 4.5 cc. of cystine, 200 p.p.m. (0.9 mg. of cystine), plus 0.5 cc. of cysteine, 200 p.p.m. (0.1 mg. of cysteine), a total of 5.0 cc., in the cyanide procedure gave a cystine value of 1.081 mg. A similar mixture in the amalgam-cyanide procedure gave a cystine value of 0.98 mg.

$$\begin{array}{rcl} 1.081 - 0.980 & = & 0.101 \text{ mg. cystine present, } 101\% \text{ of theory} \\ 0.980 - 0.101 & = & 0.879 \text{ " cysteine " } 97.7\% \text{ " " } \end{array}$$

In Table II are given the results of analyses of a series of mixtures of cystine and cysteine. Each figure is the mean of three or four determinations at the concentration given.

SUMMARY

Cystine and cysteine when estimated by the cyanide-cystine procedure are equivalent mole for mole in chromogenic value; any variation observed is due to impurity in the cysteine, irregularity in water content, or to oxidation. Cystine and cysteine when estimated by the amalgam-cyanide procedure are equivalent in chromogenic value mg. for mg., since 1 mole of cystine gives 2 moles of cysteine.

With proper attention to the purity of the cystine and cysteine standards, it is relatively easy to estimate cystine or cysteine quantitatively, singly or in mixtures.

BIBLIOGRAPHY

1. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **41**, 1030 (1926).
2. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, **44**, 1421 (1929).
3. Sullivan, M. X., *Pub. Health Rep., U. S. P. H. S.*, suppl. 78 (1929).
4. Sullivan, M. X., and Hess, W. C., *J. Biol. Chem.*, **117**, 423 (1937).
5. Pulewka and Winzer, *Arch. Exp. Path. u. Pharmacol.*, **138**, 154 (1929).
6. Shinohara, K., *J. Biol. Chem.*, **112**, 671 (1935-36).
7. Jorissen, W. P., Bassett, H., Damiens, A., Fichter, F., and Rémy, H., *J. Am. Chem. Soc.*, **63**, 889 (1941).
8. Noyes, A. A., and Steinour, H. H., *J. Am. Chem. Soc.*, **51**, 1409 (1929).

² Jorissen *et al.* (7) suggest that a better name for sodium hyposulfite ($\text{Na}_2\text{S}_2\text{O}_4$) would be sodium dithionite, first recommended by Noyes and Steinour (8).

ON THE UTILIZATION OF ACETIC ACID FOR CHOLESTEROL FORMATION*

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The information at present available as to the metabolism of cholesterol and its relationship to the other biologically important sterols is meager. Balance experiments (1) have demonstrated that cholesterol can be synthesized and destroyed in the animal but have given no convincing evidence as to the nature of specific precursors of cholesterol.

In general, balance experiments fail to differentiate between two alternatives, direct utilization of the dietary component or stimulation of a specific process of metabolism. It cannot, for example, be decided whether the increase of cholesterol observed in some experiments after the feeding of fatty substances is the result of conversion of these substances to cholesterol or whether it merely reflects an increased metabolic activity of the organism. An experiment (2) on the rate of formation of cholesterol, carried out with the aid of deuterium, indicated that about half of all the hydrogen atoms of the newly formed cholesterol was derived from the hydrogen of water; the other half must have originated from the carbon-bound hydrogen atoms of some dietary constituent. This evidence eliminated large molecules as immediate precursors, and suggested that "cholesterol... is formed by the coupling of smaller molecules, possibly those which have been postulated to be intermediates in the fat and carbohydrate metabolism" (2).

The recent observation (3) that the ingestion of deuterio acetic acid, CD_3COOH , leads to the formation of deuterio cholesterol supports this view. On the other hand, deuterio cholesterol is not formed after α,β -dideuterio propionic acid and deuterio succinic acid are fed. α,β -Dideuterio butyric acid and β,γ -dideuterio butyric acid were only slightly effective. The deuterium concentrations in the total cholesterol of the experimental animals after the feeding of deuterio acetate for 8 days are given in Table I. Positive evidence for the utilization of acetate for cholesterol formation was found for mice, growing rats, and adult rats. In each case the concentration of deuterium in the cholesterol was over 3 times that in the body water. The figures in the seventh column give the proportion

* This work was carried out with the aid of grants from the Rockefeller Foundation and the Josiah Macy, Jr., Foundation.

of all the hydrogen in the cholesterol which was derived from the dietary acetate during the experimental period. These values must of necessity be small, if only for the reason that the rate of synthesis of cholesterol is slow.

In considering possible steps in the transformation of acetate to sterol, a mechanism must be postulated in which the acetate molecules are reductively coupled to form larger units. Intermediates containing only labile hydrogen, *i.e.* readily exchangeable hydrogen, can be ruled out. For example, a pathway via oxalacetic acid cannot be considered, as it would result in the elimination of deuterium by enolization. We have tested some compounds into which acetic acid is known to be converted *in vivo* in order to determine whether any of these reactions is involved in sterol synthesis.

TABLE I
Feeding of Deuterio Acetic Acid

Experiment	Animals used	Deuterio acetic acid fed per day per 100 gm. body weight	Deuterium in			Hydrogen in cholesterol derived from acetate	Deuterium in total fatty acids
			Sodium acetate	Body water	Isolated cholesterol		
		mg.	atom per cent excess	atom per cent excess	atom per cent excess	per cent	atom per cent excess
A	Adult mice	372	9.9	0.04	0.13	1.3	0.02
B	Young rats	82	68.0	0.08	0.27	0.4	0.04
C	Adult "	97	27.6	0.05	0.21*	0.8	

* In this experiment the cholesterol was converted to cholesteryl chloride, which was analyzed.

Yeast dehydrogenases rapidly convert acetate to succinate (4). As the results obtained on feeding deuterio succinate were negative, the pathway from acetate to cholesterol does not go through succinic acid.

A conceivable explanation for the failure of succinate to generate deuterio cholesterol might be that the succinic-fumaric-malic equilibrium causes a rapid loss of deuterium to the body fluids and therefore renders detection of cholesterol formation impossible. However, this cannot be the reason. Assuming that acetate were converted to succinate and that the latter lost deuterium by exchange, then deuterio acetate should not be more effective in forming deuterio cholesterol than deuterio succinate. Succinate does not represent an intermediate step.

Little is known of acetate metabolism in normal animals. It has apparently been believed, but not proved, that acetic acid is directly burned to carbon dioxide and water at a rapid rate. Under certain conditions, such as in fasting animals (5) or in liver slices (6), formation of aceto-

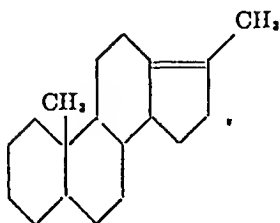
acetic acid and β -hydroxybutyric acid from acetic acid has been demonstrated and ascribed to condensation of acetic acid with either pyruvic acid (7) or another molecule of acetic acid (8).

Morehouse (9) has isolated deuterio β -hydroxybutyric acid from the urine of fasting rats which had been given β, γ -dideuterio butyric acid, but found normal β -hydroxybutyric acid after α, β -dideuterio butyric acid was fed. This demonstrates that, while the α - and β -hydrogen atoms are rapidly replaced, the γ -hydrogen atoms are relatively stable. β, γ -Dideuterio butyric acid can be expected to give rise to deuterio acetoacetic acid in normal animals also. When its sodium salt was fed to rats, the isotopic content of the cholesterol was only slightly higher than that of the body fluids, in contrast to a comparable feeding of deuterio acetate, when the cholesterol formed contained 3 to 4 times more deuterium than the surrounding body water. In view of the known relationship of butyric, acetoacetic, and β -hydroxybutyric acids these compounds may be ruled out as intermediates in sterol formation from acetic acid.

In another experiment our animals received a corresponding amount of α, β -dideuterio butyric acid. This compound was expected to exchange its deuterium by enolization after being oxidized to acetoacetic acid, and the cholesterol isolated should not contain significant concentrations of deuterium. The concentration of deuterium in the isolated cholesterol was slightly higher than that of the body fluids.

Propionic acid was tested as a possible cholesterol precursor, because of its close biological relationship to pyruvic acid. Sodium α, β -dideuterio propionate was fed to rats as a source of deuterio pyruvic acid, but failed to produce a cholesterol with a significant deuterium content. If this failure were due to removal of deuterium by enolization, the same loss of isotope should occur with acetate if it were converted to cholesterol via pyruvate. Our experimental results indicate that propionic acid and presumably pyruvic acid are not intermediates in the synthesis of cholesterol from acetate.

We have attempted, by chemical degradation of the deuterio cholesterol, to obtain some more detailed information on the mechanism of sterol synthesis. Unfortunately the yields of cholesterol fragments which could be obtained by oxidative breakdown of the available deuterio cholesterol samples would be too small to permit deuterium analysis. The only reaction found to be suitable for our purpose was the thermal degradation of cholesteryl chloride described by Mauthner (10). When this compound is heated to about 300° in a nitrogen atmosphere, hydrochloric acid is eliminated; at about 400° fission occurs between carbon atoms 17 and 20, yielding isooctane, isooctene, and a high boiling oil to which the structure shown in the accompanying formula has been assigned by Bergmann and Bergmann (11).



By converting the deuterio cholesterol isolated from our animals into cholesteryl chloride and subjecting the latter to the thermal degradation, we have obtained two fractions, representing the side chain and the sterol nucleus respectively. The concentrations of deuterium in the isooctane-isooctene mixture and in the hydrocarbon $C_{19}H_{30}$, which may be regarded as the primary degradation products, are in good agreement with that in the cholesteryl chloride. The occurrence of side reactions leading to a loss of fractions with high deuterium concentrations is therefore unlikely. Small errors in the calculation may arise from two sources. The hydrochloric acid split off in the initial phase of the degradation was not analyzed. However, unless the deuterium concentration at carbon atom 2 or 4 of the sterol nucleus was very much higher than that at other carbon atoms the analytical figure for the nucleus could not have been influenced profoundly. Secondly, the high boiling hydrocarbon $C_{19}H_{30}$ contains only one double bond instead of the two or three expected. The drastic treatment of the cholesteryl chloride evidently involves hydrogen shifts, which, however, should not greatly change the isotopic concentration of the fragments unless the deuterium concentration at any particular carbon is of a different order of magnitude from that at others.

From the analytical values obtained for the two breakdown products, it becomes evident that acetate had been utilized for the biosynthesis of the side chain as well as the steroid nucleus. Indeed the deuterium concentration found in the side chain exceeds that of the nucleus by approximately 50 per cent. The difference may arise from the fact that the methyl groups of the cholesterol at positions 18, 19, 21, 26, and 27 may originate directly from the acetic acid with the retention of all 3 hydrogen atoms, while the other carbon atoms probably retain only 1 or 2 of the original hydrogen atoms. Since the methyl groups account for 50 per cent of all the hydrogen in the side chain, and for only 20 per cent of the total hydrogen in the nucleus, more of the deuterium could be expected to be incorporated into the side chain.¹

¹ The foregoing considerations ignore the possibility that intramolecular hydrogen shift had occurred between the side chain and the nucleus during the pyrolysis. This, however, cannot be excluded at present. As the deuterium content of the isooctane is found to be higher than that of the ring hydrocarbon, the isotope must

EXPERIMENTAL

Preparation of Deuterio Compounds

Deuterio Acetic Acid—Deuterio malonic acid was prepared by exchange with heavy water and decarboxylated to deuterio acetic acid (12). For feeding experiments the sodium salt was used.

α,β -Dideuterio propionic acid was prepared from methyl acrylate as previously described (13). The sodium salt of the acid contained 34.4 atom per cent excess deuterium.

α,β -Dideuterio butyric acid was prepared by hydrogenating ethyl crotonate with deuterium gas. The sodium butyrate contained 23.1 atom per cent excess deuterium.

β,γ -Didcuterio butyric acid was similarly prepared from vinylacetic acid. The sodium salt of the β,γ -dideuterio butyric acid contained 16.0 atom per cent excess deuterium. As the β,γ double bond in vinylacetic acid is known to shift easily towards the α,β position (14), it was felt necessary to prove that the isotope was actually located at the β - and γ -carbon atoms. To 0.398 gm. of the sodium butyrate (3.57 mm) in water, 10.34 gm. (117.5 mm) of ordinary butyric acid and an excess of sulfuric acid were added. The butyric acid mixture was extracted from the aqueous solution by ether and the ether dried and distilled off. The butyric acid thus obtained was brominated according to the method of Fischer (15). The α -bromobutyric acid obtained distilled at 120–122°, 19 mm. It contained 0.49 atom per cent excess deuterium or 16.6 per cent when calculated for the undiluted acid. This compares with a value of 16.0 per cent for the deuterium analysis of the sodium β,γ -dideuterio butyrate. As the sodium butyrate and the α -bromobutyric acid both contain 7 hydrogen atoms, the agreement between the two values demonstrates that no deuterium had been lost as the result of the bromination; *i.e.*, no shift of double bonds from the β,γ to the α,β positions had occurred during the catalytic hydrogenation of vinylacetic acid.

Isolation of Cholesterol—Cholesterol was obtained in the usual manner from the unsaponifiable fractions of the total pooled carcasses. The samples were converted into the dibromides, debrominated (16), and recrystallized. The melting points of the purified cholesterol samples ranged from 147–149°.

Cholesterol Degradation—1.3 gm. of crude sterol obtained from Experiment C, Table I, were converted into cholesteryl chloride by dissolving in

have been present in the cholesterol side chain prior to the degradation. The same, however, does not hold necessarily for the sterol nucleus. The amounts of deuterio cholesterol available at present do not permit as yet the carrying out of reactions from which the deuterium content at individual carbon atoms could be determined.

chloroform and adding 0.6 gm. of freshly distilled thionyl chloride. The mixture was refluxed for 1 hour, and the solvent and excess reagent were distilled off. The dark brown residue failed to crystallize. Purification was achieved by passing a petroleum ether solution of the crude product through a column of activated alumina. The colorless petroleum ether filtrate was evaporated and the residue crystallized from a small volume of acetone. There was obtained 0.75 gm. of cholesteryl chloride, m.p. 94–95°, containing 0.21 atom per cent excess deuterium.

$C_{27}H_{48}Cl$. Calculated, C 80.1, H 11.1; found, C 79.7, H 11.4

For the thermal degradation, 0.65 gm. of the deuteriocholesteryl chloride, diluted with an equal amount of non-isotopic cholesteryl chloride, was slowly heated to 300° in an atmosphere of nitrogen. Hydrochloric acid was evolved. The temperature was kept constant until the HCl evolution ceased (4 hours). The bath temperature was then slowly raised to about 400°. At this temperature a volatile product distilled over very slowly and was collected in a trap cooled by dry ice. About 250 mg. of a mobile distillate were obtained; it was redistilled into a second trap. The liquid was presumed to be a mixture of isooctane and isooctene. Its boiling range was 115–120°; i.e., roughly the same range as that observed by Bergmann and Bergmann (11). For deuterium analysis the liquid was volatilized directly into the combustion furnace by a slow oxygen stream. The resulting water contained 0.128 atom per cent excess deuterium. Since the deuteriocholesteryl chloride had been diluted 1:1, the actual value for the isooctane-isooctene mixture is 0.256 atom per cent excess deuterium.

The residue which remained after the pyrolysis was heated over a free flame, and a viscous yellow oil distilled over between 380–400°. An attempt to obtain a crystalline hydrocarbon from this fraction was unsuccessful. 0.50 gm. of this high boiling oil was dissolved in petroleum ether and the solution passed through a column of activated alumina. Only 10 per cent of the fraction was adsorbed. The combined filtrate and petroleum ether washings were free of pigments. The colorless oil remaining after removal of the solvent had the following composition.

$C_{18}H_{36}$. Calculated, C 88.4, H 11.6; found, C 88.3, H 11.7
 $[\alpha]_D^{25} = +30.7^\circ$ (2% in benzene)

Bergmann and Bergmann reported C 87.9, H 11.9, $[\alpha]_D = +31.4^\circ$. The hydrocarbon contained 0.089 atom per cent excess deuterium, or 0.178 per cent calculated for the undiluted starting material.

On the basis of data obtained by Bergmann and Bergmann, we assume that the composition of the volatile hydrocarbon corresponds closely to that of isooctane, C_8H_{18} . The average deuterium content of the hydrogen

in a compound composed of the fragments C_8H_{18} and $C_{19}H_{30}$, as calculated from their isotope content, would be $(18 \times 0.26 + 30 \times 0.18)/48 = 0.21$ per cent. This compares well with the value 0.21 per cent found for the deuterium content of the hydrogen in the cholesteryl chloride which had been used as a starting material.

The catalytic hydrogenation of the hydrocarbon $C_{19}H_{30}$ with platinum in acetic acid consumed only one-third of the amount of hydrogen required for one double bond. The resistance to hydrogenation is in agreement with the view of Bergmann and Bergmann that the double bond in the hydrocarbon $C_{19}H_{30}$ is located at quaternary carbon atoms.

Animal Experiments

Feeding of Deuterio Acetic Acid. Experiment A—Seven adult mice received the following diet (Stock Diet I): 50 per cent casein, 20 per cent Wesson oil, 14 per cent salt mixture (17), 16 per cent yeast. The high salt content of the diet resulted in a large water consumption and urine excretion, thereby favoring the "washing out" of heavy water from the body fluids. In addition to the stock diet, each mouse received 110 mg. of sodium deuterio acetate (9.9 atom per cent excess deuterium) per day for 8 days. At the end of the experimental period, each mouse had gained an average of 4 gm. The animals were killed, body water was distilled from the tissues, and total fatty acids and cholesterol were isolated from the animal carcasses after removal of the gastrointestinal tracts.

Experiment B—Two growing rats, weighing 102 and 110 gm. respectively, were given the carbohydrate-free Stock Diet I and in addition 137 mg. of sodium deuterio acetate (68 atom per cent excess deuterium) per animal per day over a period of 8 days. Each animal gained about 30 gm. during the feeding period.

Experiment C—Four adult rats having a combined weight of 1113 gm. received the following Stock Diet II: 86 per cent casein, 3 per cent Wesson oil, 5 per cent yeast, 5 per cent salt mixture (17), 1 per cent cod liver oil. During the 8 day feeding period, each animal received 400 mg. of sodium deuterio acetate (27.6 atom per cent excess deuterium) per day. At the end of the experimental period, the combined weight of the animals was 1196 gm. A total of 1.4 gm. of crude cholesterol was obtained from the animal carcasses.

The results of the deuterium analysis of body water, fatty acids, and cholesterol are given in Table I. The data suggest that variation of dietary composition has had little effect on the formation of deuterio cholesterol. During the experimental period, the feces were collected from the animals of Experiment C, Table I. They were ground up with anhydrous sodium sulfate, extracted with ether and acetone, and the sterols precipitated from

the unsaponifiable fraction by digitonin. If we assume that the fecal sterols were derived from the body sterols and that the deuterium concentration in the body sterols increased linearly during the experiment, then the deuterium concentration of the total fecal sterol should be half of that of the body cholesterol at the end of the experiment. The fecal sterols recovered from the digitonides contained 0.110 atom per cent excess deuterium, as compared to 0.21 atom per cent excess in the body cholesterol.

Feeding of Deuterio Succinic Acid—Nine adult mice received the carbohydrate-free Stock Diet I and a daily addition per mouse of 110 mg. of deuterio succinic acid,² containing 26.9 atom per cent excess deuterium. During the experimental period the combined weight of the mice increased from 180 to 215 gm. Body water and cholesterol from the combined carcasses contained 0.13 and 0.07 atom per cent excess deuterium respectively.

TABLE II
Feeding of Deuterio Fatty Acids to Adult Rats

1.6 m μ of the sodium salt of deuterio fatty acid were fed per day per 100 gm. of body weight in each case.

Compound fed (sodium salts)	Deuterium in com- pound fed atom per cent excess	Deuterium in		
		Body water atom per cent excess	Cholesterol atom per cent excess	Fatty acids atom per cent excess
Acetic acid	27.6	0 05	0 21	
α,β -Dideuterio propionic acid	34.4	0 05	0.02	0.01
" butyric acid	23.1	0 03	0 04	
β,γ -Dideuterio " "	16.0	0.05	0 07	0.02

Feeding of Sodium α,β -Dideuterio Propionate—Two rats having a combined weight of 297 gm. received the carbohydrate-free, low fat Stock Diet II. During the experimental period of 8 days, each animal received a daily addition of 231 mg. of sodium deuterio propionate (34.4 atom per cent excess deuterium). On the 3rd day of feeding, the rats developed a slight diarrhea, which disappeared when some bone meal was added to the diet. At the end of the experimental period, the two animals had a total weight of 310 gm. The analytical data are given in Table II.

Feeding of Sodium α,β -Dideuterio Butyrate—Two rats, having a total weight of 294 gm., were given Stock Diet II and in addition 265 mg. of sodium butyrate (23.1 atom per cent excess deuterium) per animal per day. No change of weight occurred during the 8 day feeding period. The deuterium analyses for body water and cholesterol are given in Table II.

² The deuterio succinic acid was kindly supplied by Dr. Abraham Mazur. It had been prepared from diethyl fumarate by catalytic hydrogenation with deuterium gas.

Feeding of Sodium β,γ -Dideuterio Butyrate—Two rats received Stock Diet II and in addition 265 mg. of sodium butyrate (16.0 atom per cent excess deuterium) daily per rat over a period of 8 days. The combined weight of the animals increased from 291 to 329 gm. The analytical results are presented in Table II.

DISCUSSION

In all experiments the test substance was supplied with the diet for 8 days. During this period the animals were slowly synthesizing and destroying cholesterol. The rate of these reactions for the adult mouse is about 2.5 per cent per day (2); so that at the end of the 8 day period the total cholesterol, i.e. the cholesterol actually analyzed, should represent a mixture of about 1 part of newly formed and 4 parts of preexisting cholesterol. The rate of cholesterol synthesis in the rat has not been determined but may be assumed to be of similar magnitude. We know from the experiments on the deuterium content of cholesterol of mice whose body fluids contain D_2O that the maximum concentration of deuterium in their cholesterol is about half of that of the body fluids. A level of about one-tenth is reached in 8 days. Whenever in the present experiments the concentration of deuterium in the total cholesterol is appreciably over one-tenth that of the body fluids, there is presumptive evidence for a synthesis utilizing hydrogen from a deuterio dietary constituent and not from water alone.³ The appearance of cholesterol with a concentration greater than half that of the body fluids is conclusive evidence for the utilization of the isotopic test substance in the synthesis of cholesterol, since no assumption need then be made as to the rate of this reaction. The results of the three acetic acid experiments satisfy both criteria for conversion of acetate to cholesterol. In each case the deuterium concentration is over 30 times that which could be expected to enter the cholesterol from the body fluids.

Our experimental results demonstrate clearly that the cyclopentanoperhydrophenanthrene structure can be synthesized from C_2 units. A condensation of numerous small molecules with the intermediate uptake and elimination of the elements of water, with or without reduction of double bonds, is therefore suggested. From our data the exact nature of the unit undergoing condensation cannot be stated. It seems unlikely to be the relatively inert acetic acid itself, but rather a closely related inter-

³ The deuterium concentration of the body fluids was zero at the start of the experiment and at a maximum, given in Tables I and II, at the end. It may be assumed that the major part of the acetic acid was oxidized to water and carbon dioxide. From the amounts of deuterium administered and the fluid intake of the animals it can then be calculated that the deuterium concentration in the body fluids rose for about 3 days and thereafter remained relatively constant.

mediate such as acetaldehyde,⁴ into which acetic acid might readily be converted by the organism.

The available data are not sufficient to enable one to decide whether acetic acid (or a related intermediate) forms the sole precursor for cholesterol, because it is not known to what extent the dietary deuterio acetate was diluted by acetic acid formed in the organism. At present, even the occurrence of acetic acid as such in intermediary metabolism is not definitely established. However, the animal organism is undoubtedly capable of metabolizing acetic acid. The following discussion therefore refers not only to acetic acid itself but also to closely related compounds into which acetic acid might be converted by the animal.

The minimum fraction of cholesterol derived from dietary acetate can be estimated. The deuterium concentration in the cholesterol isolated from Experiment A (Table I) amounted to 1.3 per cent of that of the administered sodium acetate. Since the cholesterol analyzed represented a mixture containing approximately one-fifth of the cholesterol newly formed during the experimental period, the percentage of hydrogen derived from acetate during this period must have been $5 \times 1.3 = 6.5$ per cent. Furthermore, we know from previous findings that one-half of the total hydrogen atoms in cholesterol had, during its synthesis, originated from the body fluids. The body water in this experiment contained only 0.04 per cent deuterium. Since the deuterium concentration of the hydrogen derived from the body fluids could not have had a measurable value (<0.005 atom per cent excess), the deuterium concentration of the cholesterol hydrogen derived from acetate must have been twice as high as 6.5 per cent. We may, therefore, conclude that a minimum of 13 per cent of all the hydrogen in the newly formed cholesterol derived from organic molecules had originated in the dietary deuterio acetic acid.

Data on acetic acid production in the rat are not available. Bernhard (19), working on sulfanilamide acetylation, observed an approximately 10-fold dilution of deuterio acetate in rabbits. It may be inferred that, if acetic acid is formed at comparable rates in the rat and in the rabbit, the deuterio acetate or related compound would have been diluted several times by the ordinary analogue before being used for cholesterol synthesis. On this basis, a substantially higher fraction than the calculated 13 per cent of the cholesterol would have been derived from acetic acid. If acetic acid is the sole precursor of cholesterol, a large daily synthesis of acetic acid to dilute the dietary acetic acid must be postulated.

⁴ MacLean and Hoffert (18) in their study on the formation of fat and sterols by aerated yeast observed an inhibition of sterol synthesis by sulfite, whereas it had no effect on fat formation. They account for the inhibition by suggesting that an aldehyde is an active metabolite in sterol synthesis.

The deuterium concentrations in cholesterol after both α,β - and β,γ -deuterio butyrate are fed are slightly higher than that of the body fluids; the deuterium of the cholesterol, therefore, cannot have been derived only from the body fluids. The results may be due to hydrolysis of acetoacetate to acetic acid, although the actual value indicates that under our experimental conditions hydrolysis of acetoacetic acid could not have been its main metabolic pathway.

The concentration of deuterium in the cholesterol of animals fed succinate seems too high to have been derived only from the body fluids. A slight conversion to acetic acid may have occurred. The essentially negative results obtained with propionic, succinic, and the butyric acids indicate that the conversion of acetate to cholesterol is a specific reaction scarcely shared by the related lower fatty acids.

The observation that in animals which had formed deuterio cholesterol from deuterio acetate the fatty acids had not taken up stably bound deuterium makes it seem unlikely that fatty acids were utilized. These results do not exclude the possibility that the carbon atoms of acetic acid are utilized for fatty acid formation. The mechanisms for these two syntheses, however, must be fundamentally different.⁴

In this connection it is of interest to consider the data reported by Sonderhoff and Thomas (20) on sterol formation by oxygenated yeast with deuterio acetate as the sole nutrient. Under their experimental conditions the unsaponifiable fraction of yeast contained roughly one-third as high a deuterium concentration as the deuterio acetate used. The acetate hydrogen did not exchange in the yeast culture and acetic acid was not formed by the cells, as the acetate recovered from the culture contained an unchanged isotope concentration. The deuterium content of the sterols was twice as high as that of the yeast fats and 20 times higher than that of the yeast carbohydrates. These authors interpret their data to indicate a rather direct utilization of acetate molecules for sterol synthesis.

A mechanism involving the condensation of a great number of acetic acid or related molecules accompanied by reduction of double bonds and uptake of hydrogen from the surrounding fluids would be in agreement with the results of Sonderhoff and Thomas for yeast as well as ours for the animal tissues. The preformed chains of the higher fatty acids, such as palmitic, stearic, or the corresponding unsaturated acids, have frequently been discussed as probable building stones for the sterol molecule (21). The evidence secured from the study of fat metabolism with the aid of deuterium points strongly against a participation in sterol synthesis of the higher fatty acids *per se*. Fecal sterols of rats which had received for 8 days an addition of deuterio palmitate to a sterol-free diet did not contain an excess of isotope (22). As the excreted sterols must have been derived from chole-

terol synthesized in the animal, the fatty acid as such could not have served as a precursor. The possibility that fatty acids are the source of acetic acid and thereby supply carbon and hydrogen for cholesterol synthesis still would not permit us to regard them as precursors in the accepted sense.

SUMMARY

1. The feeding of sodium deuterio acetate to mice and rats leads to the formation of deuterio cholesterol. By degradation of the sterol isolated from the animals, isotope was shown to be present in both the side chain and the nucleus of the cholesterol molecule.

2. A minimum of 13 per cent of the hydrogen atoms of cholesterol was derived from the acetate ion. The actual value must be higher, as the dietary acetate must have been diluted either by endogenous acetate or a closely related derivative into which the acetic acid is converted by the organism prior to utilization for sterol synthesis.

3. The experimental results exclude propionic, butyric, and succinic acids directly, and pyruvic and acetoacetic acids indirectly, as intermediates in the acetate-sterol conversion.

4. The absence of deuterium in the fatty acids of animals fed deuterio acetate is additional support for the previously expressed view that fatty acids are not directly involved in cholesterol synthesis.

BIBLIOGRAPHY

1. Bills, C. E., *Physiol. Rev.*, **15**, 23 (1935).
2. Rittenberg, D., and Schoenheimer, R., *J. Biol. Chem.*, **121**, 235 (1937).
3. Bloch, K., and Rittenberg, D., *J. Biol. Chem.*, **143**, 297 (1942).
4. Wieland, H., and Sonderhoff, R., *Ann. Chem.*, **499**, 213 (1932).
5. Swendseid, M. E., Barnes, R. H., Hemingway, A., and Nier, A. O., *J. Biol. Chem.*, **142**, 47 (1942).
6. Leloir, L. F., and Muñoz, J. M., *Biochem. J.*, **33**, 734 (1939).
7. Krebs, H. A., and Johnson, W. A., *Biochem. J.*, **31**, 772 (1937).
8. Loeb, A., *Biochem. Z.*, **47**, 118 (1912).
9. Morehouse, M. G., *J. Biol. Chem.*, **129**, 769 (1939).
10. Mauthner, J., and Suida, W., *Monatsh. Chem.*, **17**, 41 (1896).
11. Bergmann, E., and Bergmann, F., *J. Chem. Soc.*, 1019 (1939).
12. Halford, J. O., and Anderson, C. L., *J. Am. Chem. Soc.*, **58**, 736 (1936).
13. Rittenberg, D., Schoenheimer, R., and Evans, E. A., Jr., *J. Biol. Chem.*, **120**, 503 (1937).
14. Fichter, F., and Sonneborn, F., *Ber. chem. Ges.*, **35**, 938 (1902).
15. Fiseher, E., and Mouneyrat, A., *Ber. chem. Ges.*, **33**, 2387 (1900).
16. Schoenheimer, R., *J. Biol. Chem.*, **110**, 461 (1935).
17. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **15**, 311 (1913).
18. MacLean, I. S., and Hoffert, D., *Biochem. J.*, **20**, 313 (1926).
19. Bernhard, K., *Z. physiol. Chem.*, **267**, 91 (1940).
20. Sonderhoff, R., and Thomas, H., *Ann. Chem.*, **530**, 195 (1937).
21. Eckstein, H. C., and Treadwell, C. R., *J. Biol. Chem.*, **112**, 373 (1935-36).
22. Stetten, D., Jr., and Schoenheimer, R., *J. Biol. Chem.*, **133**, 347 (1940).

CONJUGATED PYRIDOXINE IN RICE BRAN CONCENTRATES

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A colorimetric method for the determination of pyridoxine has been described (1) and, following studies of the urinary elimination of unchanged pyridoxine (2, 3), it has been shown by this method that a small fraction of the vitamin is altered at the 4-hydroxymethyl group and eliminated by way of the urinary system (4). A larger fraction of the vitamin is conjugated, presumably through the 3-hydroxyl group, by man and the dog, but not by the rat (4). Since work is being discontinued at this juncture, the following evidence is presented to show that water-soluble extracts of rice bran also contain free and conjugated forms of pyridoxine.

EXPERIMENTAL

Two water-soluble rice bran concentrates were used as follows: 0.5 gm. samples in 5 cc. of water were precipitated at pH 8 with 0.5 cc. of clear, saturated basic lead acetate solution. The precipitate was removed, washed with water, and the combined supernatant fluids were adjusted to pH 3 with dilute hydrochloric acid. The solution was diluted to 20 cc. and 10 cc. were allowed to percolate at a rate of 0.5 cc. per minute through a glass column (10 mm. outside diameter) containing 100 mg. of superfiltrol diluted with 900 mg. of Decalso. The adsorbent was washed with three successive 5 cc. portions of water which contained $\frac{1}{8}$ volume of McIlvaine's citrate buffer (pH 3). The vitamin was eluted with three portions (5 cc. each) of 0.25 N sodium hydroxide. Portions of the eluate were analyzed essentially as reported (1), with and without hydrolysis (4).

The following slight modifications of the test reaction were introduced. To increase the sensitivity, only 2 volumes of the half strength reagent were used instead of the 4 volumes originally recommended. To avoid salt effects, 1.5 gm. of potassium chloride were added to the aqueous phase of the test reaction. To avoid the influence of temperature variations and age of the reagent, readings were taken at 5 minute intervals until maximum absorption was attained; readings were made in this way with a blank, to indicate 100 per cent transmittance, with a standard solution, to calibrate the instrument, and with the unknown test solution.

From the data in Table I, it appears that hydrolysis increases the free pyridoxine content of the samples. Since after hydrolysis the concentrates

were found to contain about 100 γ of total pyridoxine per gm., like concentrations of crystalline pyridoxine were added to the rice brans and recovery experiments were performed. The data shown in Table I indicate moderately satisfactory recovery of added pyridoxine. When the eluates were hydrolyzed prior to analysis, satisfactory agreement with the rat growth and yeast growth methods of assay was obtained. Thus, it may be assumed that only pyridoxine was measured by the colorimetric method. Since this agreement is attained only after hydrolysis, it would appear that

TABLE I

Pyridoxine Content of Rice Bran Concentrates

The values are expressed in micrograms per gm. of concentrate.

Sample No.	Free pyridoxine	Total pyridoxine	Recovery	
			Added	Total found
1*	56	100	100	182
	56	100	100	171
	64	110	100	208
2†	56	123	100	201
	60	125		
	50	128	100	230
	50	129	100	224
	56	132	100	202
	60	132	100	223
	66	133	100	239
		138	100	200
		138	100	236
		140		
		141	100	219

* This sample, kindly furnished by Dr. Morgareidge of the National Oil Products Company, was assayed by the rat growth method and found by him to contain 110 γ per gm. Dr. Daniel Melnick, of the Food Research Laboratories, Inc., using a modified yeast growth method of assay found 115 γ of pyridoxine per gm. This value was obtained only after hydrolysis.

† This was a commercial sample. It was analyzed colorimetrically by Dr. A. D. Emmett of Parke, Davis and Company, who found 132 γ of pyridoxine per gm.

there is in rice bran a water-soluble pyridoxine conjugate of low molecular weight which is not precipitated by protein precipitants, and which is adsorbed by acid clay and eluted much as pyridoxine is. This conjugate may well be different from the one occurring in urine as a result of the metabolism of pyridoxine. For reasons already outlined (1, 4), it would appear that pyridoxine, like thiamine pyrophosphate, may be conjugated with phosphoric acid through the 3-hydroxyl group, or a readily hydrolyzed N substituent of the pyridine nucleotide type may be present in this conjugate.

It has long been recognized that pyridoxine occurs bound to protein (5), and it has been postulated (6) that the vitamin functions as the prosthetic group of an enzymatically active protein molecule. The occurrence in nature of a water-soluble pyridoxine conjugate may suggest a mode of linkage of the vitamin to the protein.

The occurrence of a conjugated pyridoxine requires an evaluation of the various types of assay procedures in use. The conjugate which occurs in urine gives the indophenol reaction only after hydrolysis (4), and recently, Snell, Guirard, and Williams (7) using a yeast growth method (8) to measure the urinary excretion of pyridoxine also found an increased response following hydrolysis, but both the free and conjugated forms of the vitamin which occur in rice bran are measured simultaneously in the rat. The differences in analytical data reported (8) may, therefore, result in part from such differences inherent in the assay procedures used.

The assistance of Mr. Walter C. Fulmer is gratefully acknowledged.

SUMMARY

Evidence is presented to show that rice bran contains free pyridoxine and a water-soluble conjugate of low molecular weight.

BIBLIOGRAPHY

1. Scudi, J. V., *J. Biol. Chem.*, **139**, 707 (1941).
2. Scudi, J. V., Koones, H. F., and Keresztesy, J. C., *Proc. Soc. Exp. Biol. and Med.*, **43**, 118 (1940).
3. Scudi, J. V., Unna, K., and Antopol, W., *J. Biol. Chem.*, **135**, 371 (1940).
4. Scudi, J. V., Buhs, R. P., and Hood, D. B., *J. Biol. Chem.*, **142**, 323 (1942). Scudi, J. V., Bastedo, W. A., and Webb, T. J., *J. Biol. Chem.*, **136**, 399 (1940).
5. Kuhn, R., and Wendt, G., *Ber. chem. Ges.*, **71**, 780, 1118, 1534 (1938).
6. Green, D. E., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **1**, 190 (1941).
7. Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, **143**, 519 (1942).
8. Williams, R. J., Eakin, R. E., and McMahan, J. R., *Univ. Texas Pub.*, No. 4137. **24** (1941).

THE DETERMINATION OF INULIN

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The clearance of inulin has been used by various workers as a measure of glomerular filtration in the mammalian kidney (1). For this purpose the determination of the concentration of inulin in blood and urine following intravenous injection of this compound is necessary. The common colorimetric methods used for this determination were investigated to determine the one preferable for use on blood and urine samples containing inulin.

In the early work inulin was determined by measuring the difference in the reducing power before and after acid hydrolysis (2). Later methods are based upon the colorimetric determination of the fructose formed following hydrolysis of the inulin. One of these colorimetric methods was developed by van Creveld (3), and was modified by Alving, Rubin, and Miller (4) and by Coreoran and Page (5). This method involves hydrolyzing the inulin and heating the resulting solution in a boiling water bath with diphenylamine and hydrochloric acid. When alcoholic diphenylamine is used, the heating is done in sealed tubes to prevent evaporation of the solvent. If an acetic acid solution of diphenylamine is used, the heating may be done in open tubes (6). When such a procedure is preceded by preliminary treatment with yeast, this method is quite specific for inulin. The nature of the reaction which takes place is not known, but a blue, water-soluble compound is formed, the concentration of which is proportional to the amount of inulin present.

A colorimetric method which was developed by Roe (7) for the determination of fructose in blood and urine may be used for the determination of the fructose formed by the hydrolysis of inulin. This method is based upon the Seliwanoff reaction and is specific for fructose in the presence of other reducing substances commonly present in blood and urine. When this method is used, the inulin is hydrolyzed and the resulting solution is heated at 80° with resorcinol in the presence of hydrochloric acid. The intensity of the color is proportional to the amount of inulin present.

When a water solution of inulin is treated by either of these methods, the blue color formed by the diphenylamine method is more intense than is the red color formed by the resorcinol method for any given concentration of inulin. This makes the diphenylamine method the more sensitive one.

Both methods, however, produce sufficient amounts of color to permit determination of concentrations of inulin above 4.0 γ per cc. when a photoelectric colorimeter and appropriate light filters are used.

Experiments were carried out to determine whether preliminary hydrolysis of the solution containing inulin was necessary (6, 8). It was found that either the resorcinol or the diphenylamine reagent gave the same amount of color when preliminary treatment with acid was omitted as when it was carried out. This probably results, at least in part, from hydrolysis taking place during the determinations. The authors feel that it is better to omit the preliminary hydrolysis, both because it makes the method less time-consuming and because prolonged treatment with acid, if not carefully controlled, may result in destruction of fructose.

Glucose produces a measurable color by the diphenylamine reaction. The amount of this color from a 10.0 mg. per cent solution is equivalent, on the average, to that from 0.2 mg. per cent fructose. The same concentration of glucose when treated by the resorcinol method produces only a very small amount of color, the intensity of which could not be measured by the photoelectric colorimeter used in these studies. If filtrates are prepared by diluting normal blood plasma 10 times, it is therefore not necessary to remove glucose when the inulin is determined by the resorcinol method. Glucose, however, must be removed from such filtrates when the diphenylamine method is employed if a high degree of accuracy is desired.

Normal undiluted urine contains some non-fermentable substance or substances which give a color when treated by either method. It was found by experiment that the color was due to two reactions. One of these reactions was between the urine and the resorcinol and the other was between the urine and the acid which was present. The latter color can be accounted for in urine samples containing inulin by making simultaneous determinations in which the resorcinol is omitted. The former color is usually of such small amount that it is unmeasurable if the urine is diluted sufficiently when the determination is made. The amount of color varies in different samples with the concentration of the urine. In general, if the urine is diluted 100 or more times, no measurable color is present. When inulin clearance determinations are made, dilutions to this extent are necessary because of the high concentration of the polysaccharide present.

The method considered most satisfactory is described below. Although this method is not considered more accurate than the diphenylamine method, it is preferred because it is not necessary to remove glucose from the samples.

Reagents—

1. 0.1 per cent resorcinol in 95.0 per cent alcohol.
2. 30.0 per cent hydrochloric acid.
3. A stock standard inulin solution containing 1.0 mg. per cc., from which a working standard is prepared by diluting this 1:50 or 1:100 at the time when the analyses are made.
4. Somogyi zinc sulfate deproteinizing agents: (a) 12.5 gm. of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 125 cc. of 0.25 N H_2SO_4 and made up to 1000 cc. with water; (b) 0.75 N NaOH in distilled water.

Procedure for Blood—When the determination is made on blood plasma, the plasma is deproteinized according to the method of Somogyi (9), which is as follows: To 1.0 cc. of plasma are added 8.0 cc. of acid zinc sulfate (Solution I) and 1 cc. of sodium hydroxide (Solution II). After standing for $\frac{1}{2}$ hour the mixture is filtered. The filtrate is diluted so that it contains between 0.5 and 4.0 mg. per cent of inulin. To 1.0 cc. of the diluted filtrate are added 1.0 cc. of the alcoholic resorcinol and 3.0 cc. of 30.0 per cent hydrochloric acid. The solution is mixed well and placed in a water bath at 80° for exactly 8 minutes, after which it is cooled in running water. The volume is made up to 5.0 cc. with 95 per cent alcohol and the solution is mixed again. The color is compared with that given by inulin solutions which have been simultaneously treated by the same procedure. A photoelectric colorimeter having a filter with maximum transmission of light at 450 m μ was used in the present experiments.

Procedure for Urine—If a protein-free urine is used, it is only necessary to dilute the urine so that it contains between 0.5 and 4.0 mg. per cent of inulin. The urine should be diluted at least 100 times. To 1.0 cc. of the diluted urine in a test-tube are added 1.0 cc. of alcoholic resorcinol and 3.0 cc. of 30.0 per cent hydrochloric acid. To a second test-tube containing 1.0 cc. of the diluted urine are added 1.0 cc. of 95 per cent alcohol and 3.0 cc. of 30.0 per cent hydrochloric acid, without the resorcinol. The two tubes are shaken and placed in a water bath at 80° for exactly 8 minutes, together with a standard solution of pure inulin. They are then cooled in running water. The intensity of the colors is determined in the same manner as that used for blood samples.

Calculations—The calculation for the inulin content of the plasma is the same as that used for any colorimetric determination. The concentration of inulin in the urine samples is the difference between the concentration found in the tube to which resorcinol was added and that in the tube to which no resorcinol was added. The color formed in the latter tube was due to a reaction between the acid and the urine and not due to the inulin.

Blood—

$$\frac{(\text{Reading of unknown})(\text{dilution factor})}{(\text{Reading of standard 1.0 mg. \%})} = \text{mg. inulin per 100 cc.}$$

Urine—

$$\frac{(\text{Reading of unknown minus reading of acid-urine control})(\text{dilution})}{(\text{Reading of standard 1.0 mg. \%})} = \text{mg. inulin per 100 cc.}$$

In Table I a series of determinations is given showing the recovery from plasma and urine of added inulin.

TABLE I
Recovery of Added Inulin from Plasma and Urine

Plasma				Urine*			
Dilution	Inulin added	Inulin recovered	Per cent recovery	Dilution	Inulin added	Inulin recovered	Per cent recovery
	mg. per cent	mg. per cent			mg. per cent	mg. per cent	
1:10	1.0	1.02	102	1:100	1.0	1.01	101
	2.0	1.95	98		1.0	0.96	96
1:20	1.0	1.01	101	1:200	2.0	1.98	99
	3.0	3.02	101		2.0	2.02	101
1:30	1.0	1.02	102	1:300	3.0	2.85	95
	4.0	3.87	97		3.0	3.10	103
1:40	3.0	3.01	100	1:400	1.0	0.96	96
	4.0	3.88	97		3.0	3.20	107
					4.0	4.30	107
					2.0	2.01	101
					4.0	4.21	105

* If the urine is diluted less than 100 times, the apparent recovery of inulin is high.

SUMMARY

The factors which influence the colorimetric determination of inulin in blood and urine are discussed. An adaptation of the Roe method for fructose is described for the determination of inulin in blood and urine. This method does not require the preliminary removal of glucose from normal blood samples.

BIBLIOGRAPHY

1. Smith, H. W., *The physiology of the kidney*, New York (1939). Goldring, W., Chasis, H., Ranges, H. A., and Smith, H. W., *J. Clin. Inv.*, **19**, 739 (1940). Miller, B. F., Alving, A. S., and Allison, J. C., *J. Clin. Inv.*, **18**, 494 (1939). Hendrix, J. P., Westfall, B. B., and Richards, A. N., *J. Biol. Chem.*, **116**, 735 (1936).

2. Shannon, J. A., and Smith, H. W., *J. Clin. Inv.*, **14**, 393 (1935).
3. van Creveld, S., *Klin. Woch.*, **6**, 697 (1927).
4. Alving, A. S., Rubin, J., and Miller, B. F., *J. Biol. Chem.*, **127**, 609 (1939).
5. Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, **127**, 601 (1939).
6. Harrison, H. C., *Proc. Soc. Exp. Biol. and Med.*, **49**, 111 (1942).
7. Roe, J. H., *J. Biol. Chem.*, **107**, 15 (1934).
8. Steinitz, K., and Türkand, H., *J. Clin. Inv.*, **19**, 285 (1940).
9. Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).

STUDIES ON THE PHOSPHORUS COMPOUNDS OF BRAIN

III. DETERMINATIONS OF ADENOSINE TRIPHOSPHATE AND ITS DECOMPOSITION PRODUCTS IN FRESH AND AUTOLYZED DOG BRAIN

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The isolation of adenosinetriphosphoric acid from brain was reported recently (1). The present study deals with the quantitative determination of this compound in brain frozen *in situ*, and the changes it undergoes during postmortem autolysis.

Since suitable methods are not available for separating adenosine tri- and diphosphate, adenylic acid, and inosinic acid, we have confined our examinations to the amounts of (a) nucleotide, and nucleoside plus free purine, (b) acid-hydrolyzable phosphorus combined with the nucleotide, and (c) adenine and hypoxanthine in both the nucleotide and the nucleoside-free purine fractions. By means of these studies it is possible to follow the loss of phosphoric acid and of the amino group, and the conversion of nucleotide to nucleoside or free purine during the autolytic breakdown of adenosine triphosphate.

EXPERIMENTAL

Brain tissue was secured from dogs under amytal anesthesia (65 mg. per kilo injected intraperitoneally). For determination of the initial composition, the brain was frozen *in situ* with liquid air and sampled as previously described (2), except that artificial respiration was not used. For a study of the composition after short periods of autolysis (30 seconds or less) the brain was removed from the animal, thrown into a mortar containing liquid air, and immediately flattened with a potato masher to permit more rapid cooling. The frozen tissue was ground to a powder, transferred to a tared flask containing 10 per cent trichloroacetic acid, and reweighed. For periods of autolysis of more than 30 seconds the brain was weighed rapidly to the nearest decigram, then ground in a mortar with a measured portion of iced trichloroacetic acid. The period of autolysis was measured from the time the brain was excised to the moment when it was immersed in

liquid air or crushed in acid. Extra trichloroacetic acid was added to give a mixture of 1 part of brain to 10 of fluid (brain water included) and a concentration of 10 per cent acid. After standing for 20 minutes with occasional mixing, the ice-cold mixture was filtered, and the filtrate was measured and neutralized to phenolphthalein with concentrated NaOH solution.

Methods of Analysis—Total purine nucleotide, nucleoside plus free purine, and the amounts of adenine and hypoxanthine in the nucleotide fraction were measured by procedures previously described (3-6). The term acid-hydrolyzable phosphorus refers to the amount of organic phosphorus hydrolyzed on being heated at 100° in the presence of 1 N HCl. Unless otherwise indicated, the period of hydrolysis was 15 minutes, in which 67.2 to 67.9 per cent of adenosine triphosphate phosphorus is hydrolyzed (7). After hydrolysis the liberated phosphoric acid was determined by the method of Fiske and Subbarow (8). Organic acid-hydrolyzable phosphorus equals the difference between the value thus obtained and the sum of inorganic and phosphocreatine phosphorus (9). "Stable" nucleotide phosphorus may be calculated from the values obtained for adenine and hypoxanthine, on the basis of 1 mole of phosphorus per mole of purine.

It is important to know to what extent the determination of acid-hydrolyzable organic phosphorus in the original trichloroacetic acid filtrate represents the so called pyrophosphate fraction of adenosine triphosphate; hence for the measurement of this fraction it is desirable to separate the adenosine triphosphate from the solution. With this in view Stone (10) precipitated with calcium hydroxide and considered the separation to be quantitative because no loss occurred on dissolving and reprecipitating. We find, however, that the amount of acid-hydrolyzable organic phosphorus precipitated under the conditions described by Stone is considerably less than that precipitated by mercuric acetate, in one case being only 76 per cent of the latter value. If the filtrate from the calcium hydroxide precipitate is made 0.2 per cent acid with acetic acid and treated with mercuric acetate, an additional quantity of acid-hydrolyzable phosphorus is precipitated together with purine, in the ratio of 5 atoms of N to from 0.7 to 1.2 of hydrolyzable P, with the latter representing 38 to 54 per cent of the total phosphorus. The material in the calcium hydroxide filtrate may be either adenosine diphosphate or a mixture of the triphosphate, diphosphate, and adenylic acid. We suspect it to be the latter, for calcium hydroxide precipitates only 80 per cent of pure adenosinetriphosphoric acid under the conditions used by Stone (*i.e.*, concentrations of nucleotide and of trichloroacetic acid similar to those found in protein-free brain filtrates). When the trichloroacetic acid is omitted, practically none of the compound is precipitated. This is also the case when the mixture of adenosinetriphosphoric acid and trichloroacetic acid is first neutralized with NaOH

and subsequently treated with calcium hydroxide. It appears certain that not all of the acid-hydrolyzable phosphorus bound to nucleotide is precipitated by calcium hydroxide. This explains Stone's observation that in the

TABLE I

Adenine and Hypoxanthine (or Guanine) Nucleotide, Nucleoside Plus Free Purine, and Organic Acid-Hydrolyzable Phosphorus in Fresh and Autolyzed Dog Brain (Not Crushed); Comparison of Determinations on Trichloroacetic Acid Extract and on Mercuric Acetate Precipitate

The results are expressed in mg. of N or P per 100 gm. of brain.

Experiment No.	Duration of autolysis	Trichloroacetic acid filtrate					Mercuric acetate ppt.				
		Nucleotide N	Adenine N in nucleotide	Hypoxanthine (or guanine) N in nucleotide	Nucleoside + free purine N	Organic acid-hydrolyzable P	Total purine N	Nucleotide N	Nucleoside + free purine N (by difference)	Organic acid-hydrolyzable P	Molar ratio, acid-hydrolyzable P to nucleotide
	sec.										
32	0	21.2	17.3	2.2	2.0	19.3	21.8	20.0	1.8	17.9	2.04
18	0	19.0	16.4	2.6	2.9	18.2	19.9	18.7	1.2	17.2	1.98
19	0	19.0	15.4	2.8	2.6	16.2	20.5	19.2	1.3	15.6	1.86
21	0	18.4			1.4	17.2	19.4	19.1	0.3	17.8	
22	0	17.3	14.7	1.9	1.3	16.4	18.8	17.3	1.5	15.5	2.05
Average.....		19.0	16.0	2.4	2.0	17.5	20.1	18.9	1.2	16.8	1.98
	min.										
28	2	19.8	15.6	2.1	2.1	16.4	21.2	18.4	2.8	15.0	1.85
33	5	19.2	16.6	2.1	2.3	14.5	19.4	18.9	0.5	13.7	1.61
30	10	19.3	15.6	2.0	2.4	14.5	20.9	18.5	2.4	13.7	1.70
29	20	17.3	12.1	2.0	2.6	13.0	19.9	17.3	2.6	12.2	1.88
34	30	19.1	16.5	2.4	2.1	14.1	21.2	19.4	1.8	13.2	1.53
27	40	19.8	16.3	2.0	2.9	13.7	21.0	19.8	1.2	12.3	1.47
25	1	17.9	15.1	2.5	3.0	12.6	21.7	16.9	4.8	11.2	1.39
26	1	19.1	15.5	2.6	3.6	12.5	22.0	19.5	3.5	12.0	1.45
36	2	18.2	15.4	2.6	3.5	10.0	23.0	18.1	4.9	9.1	1.10
24	3	15.8	13.3	2.3	2.0	8.1	17.9	14.3	3.6	7.7	1.07
37	5	17.9	13.4	2.8	5.5	6.2	22.7	16.0	6.7	5.5	0.72
35B	10	13.7	9.5	2.4	6.0	5.1				3.7	0.66
38	20	14.5	10.0	2.2	7.4	3.5	20.8	14.2	6.6	2.3	0.41
17	35	11.2	7.3	2.2	11.1	4.5	17.7			2.0	0.45
39	60	10.1	6.0	2.0	8.8	2.9	17.3	10.9	6.4	1.3	0.34

calcium hydroxide filtrate (which he designates the hexose phosphate fraction) 28 to 48 per cent of the phosphorus is hydrolyzed in 7 minutes in N HCl at 100°. In a similar filtrate from the mercuric acetate precipitate only 8 per cent of the organic phosphorus is hydrolyzed in this time.

Evidence that mercuric acetate in the presence of 0.2 per cent acetic acid precipitates the nucleotides of brain quantitatively is presented in Table I. The average value for nucleotide nitrogen in the eighteen experiments in which a comparison is possible was 17.9 mg. per 100 gm. precipitated by uranium acetate from the original trichloroacetic acid filtrate, while 17.6 mg. per cent were found in the mercuric acetate precipitate. It has already been shown that uranium acetate precipitates the purine nucleotides quantitatively, but does not precipitate nucleosides or free purines (3, 11, 12). The data in Table I show that mercuric acetate precipitates not only all of the nucleotide but also the greater part of the nucleoside and free purine fraction of brain. This is not true for muscle filtrates. The reason for this interesting difference has not yet been determined.

Since the mercuric acetate precipitate contains all of the nucleotide, it must also contain all of the acid-hydrolyzable organic phosphorus combined with the nucleotide. The amount of acid-hydrolyzable phosphorus thus precipitated represents about 96 per cent (average for five experiments) of that found in the original filtrate when the brain is frozen in liquid air, but as low as 44.5 per cent with autolyzed brain (Table I), the difference between the two values remaining approximately the same (average for fifteen experiments 1.1 mg. per 100 gm.). Correction of the 15 minute value by subtracting the amount of phosphorus hydrolyzed in a second 15 minute period results in a closer correspondence only for brain which has not been subjected to autolysis.

Mercuric Acetate Precipitation Procedure—A 100 cc. portion of the neutralized trichloroacetic acid extract (representing 10 gm. of brain) is acidified with acetic acid to a concentration of 0.1 per cent; then 5 cc. of 20 per cent mercuric acetate dissolved in 2 per cent acetic acid are added, the acid concentration thus becoming 0.2 per cent. After the mixture has stood on ice until sedimentation is complete, the precipitate is centrifuged, washed once with 0.5 per cent mercuric acetate, and then brought into suspension by shaking with 20 cc. of water, after which the stopper and walls of the tube are rinsed, the tube is immersed in ice, and H_2S is passed through the suspension for 45 minutes. After removal of H_2S by aeration the mixture is diluted to 50 cc. and filtered through a dry filter. Suitable portions of the filtrate are taken for determinations of total, acid-hydrolyzable, and inorganic phosphorus. Purine nucleotides are precipitated by uranium acetate from 5 cc. portions of the filtrate and determined as previously described (3). For the determination of total purine nitrogen 5 cc. aliquots are hydrolyzed for 20 minutes at 100° in 1 N H_2SO_4 (precipitation by uranium acetate being omitted), and the purines are precipitated with cupric hydroxide and then as the cuprous-bisulfite complex as in the usual procedure (3). Separate determinations of adenine and hypoxanthine may

be made by the macroprocedure described for nucleotides (3) if a larger portion of the trichloroacetic acid filtrate is taken for precipitation by mercuric acetate. The nucleosides and free purines contained in the filtrate from the uranium nucleotide may then also be studied. The nucleosides are first hydrolyzed in boiling normal acid, uranium is removed by neutralization, and the purines are precipitated as the cuprous-bisulfite complex and analyzed by the methods of Hitchings (3-6).

Results

The values for nucleotide, nucleoside plus free purine, and the amounts of adenine and hypoxanthine in the nucleotide fraction, all determined on the trichloroacetic acid filtrate, are presented in Table I. Nucleotide and total purine determined in the mercuric acetate precipitate as well as the organic acid-hydrolyzable phosphorus in this fraction are also reported.

In the dog brain frozen *in situ* with liquid air, 19 mg. of nucleotide nitrogen per 100 gm. are found, 83.5 per cent of this (average) being adenine.¹ Only traces of uric acid were detected. The molar ratio of organic acid-hydrolyzable phosphorus to nucleotide is close to that required if all nucleotide were present as nucleoside triphosphate (Table I). This ratio was calculated from the values for hydrolyzable organic phosphorus in the mercuric acetate precipitate, and the sum of adenine and hypoxanthine precipitated by uranium acetate. The fact that guanine is included with the hypoxanthine does not affect the molar ratio, unless the ratio of silver to purine proves to be different from that in the argentipicrate of hypoxanthine.

Phosphorus attached to the purine nucleosides (*i.e.*, the sum of organic acid-hydrolyzable phosphorus and the calculated value for stable nucleotide phosphorus) amounts to 25.2 mg. (average), or 36 per cent of the total acid-soluble phosphorus, the latter value being 69.2 mg. per 100 gm. (average for the five experiments on brain frozen *in situ*). Nucleoside plus free purine nitrogen averages 2.0 mg. per 100 gm. in the fresh brain, or 9.5 per cent of the total acid-soluble purine nitrogen.

¹ In a recent publication (6) Hitchings states that guanine, if present, would be precipitated together with the hypoxanthine as an insoluble silver salt. All of the experiments recorded in this paper were completed before this publication appeared; hence the values recorded for hypoxanthine include guanine. If the ratio of silver to guanine is similar to that for hypoxanthine, our results can be recalculated to moles of purine, since the silver in the precipitate was measured and the nitrogen equivalent calculated. We have made two determinations of guanine in the nucleotide fraction by the method of Hitchings (6), finding 2.7 mg. of guanine nitrogen per 100 gm. of brain 30 seconds after excision, and 2.1 mg. after 30 minutes. It seems, therefore, that the major part, if not all, of the purine in the filtrate from the adenine picrate is guanine and not hypoxanthine.

During autolysis the first change noted is a decrease in the organic acid-hydrolyzable phosphorus. This occurs within 5 seconds after removal of the brain, and continues progressively as autolysis proceeds, the value becoming approximately half of that required for full phosphorylation in about 3 minutes. Nucleotide nitrogen, which does not change during this interval, then begins a gradual decline, reaching half the normal level within 1 hour, while nucleoside and free purine increase simultaneously at the

TABLE II

Distribution of Purines in Nucleoside-Free Purine Fraction in Dog Brain after Autolysis; Analyses on Mercuric Acetate Precipitate

The results are expressed in mg. per 100 gm. of brain.

Duration of autolysis	Nucleoside + free purine N	Adenine N	Hypoxanthine N	Ribose
<i>min.</i>				
15	8.2	3.5	2.0	7
35	7.1	2.9	3.4	8
60	6.4	1.7	4.9	4

TABLE III

Changes in Purine Nucleotides and Acid-Hydrolyzable Organic Phosphorus of Brain (Dog) Crushed in Water

The results are expressed in mg. per 100 gm. of brain.

Experiment No.	Duration of autolysis	Trichloroacetic acid filtrate					Mercuric acetate ppt.			
		Nucleotide N	Adenine N in nucleotide	Hypoxanthine N in nucleotide	Nucleoside and free purine N	Total purine N (by addition)	Total purine N (determined)	Nucleotide N	Nucleoside and free purine N (by difference)	Acid-hydrolyzable organic P
	<i>min.</i>									
41	1	18.8	12.0	Lost	4.0	22.8	22.3	17.0	5.3	9.1
42	5	10.6	6.7	2.0	7.3	17.9	18.0	11.4	6.6	2.7
35A	10	8.7	5.5	2.4	8.1	16.8				2.4
43	30	4.2	0	2.8	12.7	16.9	16.1	4.9	11.2	1.3

expense of nucleotide. The decrease of nucleotide is accounted for entirely in the adenine fraction.

During 1 hour of autolysis no accumulation of inosinic acid takes place, the value determined for hypoxanthine in the nucleotide fraction remaining close to that found in fresh brain.

The fraction containing nucleoside and free purine was examined for its content of adenine and hypoxanthine in three experiments. The mercuric acetate precipitate prepared from a trichloroacetic acid filtrate equivalent

to 50 gm. of brain was used for this study. After removal of nucleotides and of uranium a portion of the filtrate tested with Bial's orcinol reagent was found to contain pentose. The green color was compared with arabinose standards similarly treated as a rough measure of the ribose content. The purines in the remaining solution were precipitated and analyzed by the method of Hitchings (3-6). The results are presented in Table II. Both adenine and hypoxanthine are found in the nucleoside-free purine fraction, together with enough ribose to account roughly for the adenine or part of the hypoxanthine (but not both) as nucleoside.

Autolysis for a period of 1 hour causes no significant change in the total purine nitrogen other than that expected from deamination of adenine.

When the brain is crushed in water and then allowed to stand, the autolytic changes are more rapid, adenine nucleotide decreasing in 5 minutes to a level requiring 30 minutes in intact brain. Within 30 minutes the adenine nucleotide disappears completely (Table III).

The difference between total purine and nucleotide nitrogen in the mercuric acetate precipitate is presented for the sake of comparison with the nucleoside and free purine fraction as determined in the trichloroacetic acid filtrate (Table I). The comparison shows that mercuric acetate precipitates the major portion of the nucleoside-free purine fraction in brain extracts provided autolysis has not proceeded longer than 20 minutes.

DISCUSSION

The pathway of autolytic decomposition of adenylic acid appears to vary in different tissues. In frog muscle ground with water Parnas (13) found adenylic acid deaminized with no further decomposition of the inosinic acid thus produced. Others (14-16) found varying amounts of nucleoside and free purine in autolyzing rabbit and beef muscle, Ostern alone (14) finding no adenine present in this fraction. Mozolowski (16) concluded that adenine is absent from the nucleoside-free purine fraction of blood. His tabulated data, however, record significant quantities (21 and 31 per cent of the total purine nitrogen in the uranium filtrate) in two of his three experiments.

In brain we find no accumulation whatever of inosinic acid as adenylic acid disappears, but rather a decomposition to nucleoside and free purine, adenine being found in this fraction and decreasing progressively during autolysis. The question is whether, after two phosphoric acid groups have been liberated from adenosinetriphosphoric acid, the adenylic acid thus set free is next deaminized to form inosinic acid, or dephosphorylated with formation of adenosine, the latter being subsequently deaminized. Enzymes are present, at least in muscle, for deaminizing both adenylic acid and adenosine, Schmidt (17) having found separate deaminases for the two

compounds. A phosphatase specific for the purine ribonucleotides and designated 5-nucleotidase has been reported by Reis (18), who noted that mammalian muscle contains little, frog muscle none, and nerve tissue relatively large quantities of this enzyme. The difference in the behavior of adenylic acid on autolysis in the tissues of various species finds an explanation in the distribution of this nucleotidase. Since frog muscle contains none of the enzyme, the nucleotide is deaminized but not further decomposed. In brain, however, which is rich in the 5-nucleotidase, the adenylic acid is split to nucleoside and free purine. In rabbit muscle, which contains little of the enzyme, small amounts of nucleoside and free purine are formed. Pohle (19), however, found no nucleotide remaining in rabbit muscle incubated 4 hours in 2 per cent sodium bicarbonate solution. In the nucleoside fraction he found carnine, and this was increased at the expense of adenylic acid when the latter was added to the digestion mixture.

The progressive decrease of adenine in the fraction containing nucleoside and free purine, together with the absence of enzymes capable of deaminizing adenine (17, 20, 21), indicates that the adenylic acid is dephosphorylated before being deaminized. If the pathway of decomposition were by way of inosinic acid, no adenine should be found in the nucleoside-free purine fraction. A similar conclusion was reached by von Euler and Skarzynski (22) for the pathway of decomposition of adenylic acid in embryonic tissue, liver, and Jensen sarcoma of rats.

The fact that the decomposition of nucleotide to nucleoside or free purine begins only after the acid-hydrolyzable phosphorus has decreased to a level equal to the stable nucleotide phosphorus indicates that the nucleotidase which splits phosphorus from adenylic acid remains ineffective as long as the nucleoside is in the form of di- or triphosphate, a condition which also prevents the deamination of adenylic acid (23). This is in harmony with the observation of Reis (18) that the nucleotidase has no effect on adenosine triphosphate, but only on adenylic and inosinic acids.

The molar ratio of acid-hydrolyzable phosphorus to nucleotide purine indicates the attachment of three phosphoric acid groups to each molecule of purine nucleoside in brain frozen *in situ*. Since only 83.5 per cent of the purine in this fraction can be accounted for as adenine, the remainder (hypoxanthine or guanine) must also be present as the triphosphate. The presence of inosine triphosphate in resting brain is difficult to accept in the absence of any increase of hypoxanthine in the nucleotide fraction during autolysis, and also because of the conclusion of Embden and Schmidt (23) that deamination of adenine does not occur as long as it is present as adenosine triphosphate. These difficulties are removed by our observation¹ that most if not all of the nucleotide purine other than adenine is

actually guanine, and it appears likely that the guanine is present as guanosine triphosphate.

It is of interest to note that the decomposition of nucleotide begins at the time free sugar has disappeared, about 3 minutes after removal of the brain (24).

No appreciable liberation of ammonia is to be expected from brain within 2 minutes after death, according to our experimental data; then a slow production should occur as adenylic acid and adenosine are decomposed. In brain allowed to stand after being crushed in water, the maximum yield of ammonia nitrogen from this source would be about 3 mg. within 30 minutes, since all of the 16 mg. of adenine nucleotide nitrogen is deaminized in this period (Table III). In a similar period in uncrushed brain less than 2 mg. of ammonia N would be liberated (Table I). These calculations are of especial interest in view of the values reported in the literature for brain ammonia (25-27). These vary from 0.1 to 2.8 mg. of N per 100 gm. On incubating brain with 2 per cent NaHCO_3 for 2 hours, Riebeling (25) found values of between 8 and 10 mg. of ammonia nitrogen per 100 gm., not more than 3 mg. of which could have originated in adenine according to our data. Preliminary experiments conducted in this laboratory have revealed major sources of error in the methods used in some of these investigations, and show that the ammonia nitrogen of brain frozen *in situ* is less than 0.3 to 0.5 mg. per 100 gm.²

SUMMARY

Methods are described for analysis of the purines and the acid-hydrolyzable phosphorus attached to the nucleotide after separation of the nucleotides from the trichloroacetic acid filtrate by precipitation with mercuric acetate.

Dog brain frozen *in situ* with liquid air contains 19 mg. of acid-soluble purine nucleotide and 2 mg. of nucleoside and free purine nitrogen per 100 gm. Of the nucleotide nitrogen, 83.5 per cent is adenine. Preliminary studies indicate that the remainder is guanine rather than hypoxanthine. The amount of acid-hydrolyzable phosphorus found attached to the nucleotide is close to that required for full phosphorylation.

During autolysis loss of acid-hydrolyzable phosphorus begins within 5 seconds, reaching a level corresponding to adenosine diphosphate in about 3 minutes, after which the nucleotide nitrogen slowly decreases to half the normal level within an hour, with a corresponding increase of nucleoside and free purine. No formation of inosinic acid is observed during 1 hour of autolysis. Adenine together with hypoxanthine and ribose is found in the nucleoside-free purine fraction, the adenine decreasing during autolysis.

² Fawaz, G., unpublished experiments.

The decomposition of nucleotide to nucleoside and free purine with a gradual decrease of adenine in the nucleoside fraction and no increase of inosinic acid suggests that adenylic acid in brain is first decomposed by a phosphatase rather than by a deaminase.

BIBLIOGRAPHY

1. Kerr, S. E., *J. Biol. Chem.*, **140**, 77 (1941).
2. Kerr, S. E., *J. Biol. Chem.*, **110**, 625 (1935).
3. Kerr, S. E., *J. Biol. Chem.*, **132**, 147 (1940).
4. Hitchings, G. H., Dissertation, Harvard University (1933).
5. Hitchings, G. H., and Fiske, C. H., *J. Biol. Chem.*, **140**, 491 (1941); **141**, 827 (1941).
6. Hitchings, G. H., *J. Biol. Chem.*, **143**, 43 (1942).
7. Kerr, S. E., *J. Biol. Chem.*, **139**, 121 (1941).
8. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).
9. Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
10. Stone, W. E., *J. Biol. Chem.*, **135**, 43 (1940).
11. Kerr, S. E., and Blish, M. E., *J. Biol. Chem.*, **98**, 193 (1932).
12. Thannhauser, S. J., and Czoniczer, G., *Z. physiol. Chem.*, **110**, 307 (1920).
13. Parnas, J. K., *Biochem. Z.*, **206**, 16 (1929).
14. Ostern, P., *Biochem. Z.*, **221**, 64 (1930).
15. Dmochowski, A., *Biochem. J.*, **23**, 1346 (1929).
16. Mozolowski, W., *Biochem. Z.*, **206**, 150 (1929).
17. Schmidt, G., *Z. physiol. Chem.*, **179**, 243 (1928).
18. Reis, J., *Enzymologia*, **2**, 110, 183 (1937).
19. Pohle, K., *Z. physiol. Chem.*, **185**, 9 (1929).
20. Winternitz, M. C., and Jones, W., *Z. physiol. Chem.*, **60**, 180 (1909).
21. Leonard, V. N., and Jones, W., *J. Biol. Chem.*, **6**, 453 (1909).
22. von Euler, H., and Skarzynski, B., *Z. physiol. Chem.*, **263**, 259 (1940).
23. Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **186**, 205 (1930).
24. Kerr, S. E., and Ghantus, M., *J. Biol. Chem.*, **117**, 217 (1937).
25. Riebeling, C., *Klin. Woch.*, **10**, 554 (1931); **13**, 1422 (1934).
26. Schwarz, H., and Dibold, H., *Biochem. Z.*, **251**, 190 (1932).
27. Bülow, M., and Holmes, E. G., *Biochem. Z.*, **245**, 459 (1932).

TRYPTOPHANE METABOLISM

X. THE EFFECT OF FEEDING *l*(-), *dl*-, AND *d*(+)-TRYPTOPHANE, *d*(-)-AND *dl*- β -3-INDOLELACTIC ACID, β -3-INDOLEPYRUVIC ACID, AND *l*(-)-KYNURENINE UPON THE STORAGE OF LIVER GLYCOGEN AND THE URINARY OUTPUT OF KYNURENIC ACID, KYNURENINE, AND TOTAL ACETONE BODIES*

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The amount of tryptophane required for maintenance and growth is small (2). When an excess is supplied to some animals, kynurenic acid is excreted (3) and, under certain conditions, kynurenine also (4). Although these are quantitatively the most important of the known metabolic products of tryptophane, the amounts excreted in extensive tests in the dog and rabbit have usually accounted for less than half, more often for less than a third, of the tryptophane administered (4-6).¹

Whether tryptophane is glycogenic or ketogenic is not clear. A number of years ago Dakin (7) observed that the injection of 14.5 gm. of *l*(-)-tryptophane into the phlorhizinized dog yielded 2.7 gm. of extra glucose, 3 gm. of kynurenic acid, and a precipitate with mercuric sulfate equivalent to about 3 gm. of tryptophane. He considered the yield of glucose too small to warrant concluding that it was formed from tryptophane. Perfusion of a surviving liver with 1.5 gm. of *l*(-)-tryptophane yielded no acetoacetic acid or acetone.

The studies recorded in this communication were directed toward determining whether a change could be shown in acetone body output upon feeding tryptophane to the fasted, but otherwise normal, rat, or to the rat fed sodium butyrate, and whether such feeding would affect the storage of liver glycogen. We were also interested in determining how the *dl* and

* The experimental data in this paper are taken from a dissertation submitted by Raymond Borchers in May, 1942, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry in the Graduate College of the State University of Iowa. A preliminary report has been published (1).

Some of the procedures employed are based on data from a thesis submitted by Newton E. Whitman for the degree of Master of Science in August, 1939.

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¹ Berg, C. P., and Rowe, V. K., unpublished data.

d(+) forms of tryptophane, indolepyruvic, *dl*- and *d*(-)-indolelactic acids, and *l*(-)-kynurenine would compare with *l*(-)-tryptophane in these respects and in their effect upon the excretion of kynurenine and kynurenic acid in this species.

EXPERIMENTAL

The compounds tested were prepared by methods previously employed in this laboratory. Total nitrogen was determined by a semimicro-

TABLE I
Physical Properties of Compounds Studied

Compound	Melting point		Total nitrogen		$[\alpha]_D^{25}$ found	$[\alpha]_D^{25}$ recorded
	Found (uncorrected)	Recorded	Found	Calculated		
	°C.	°C.	per cent	per cent	degrees	degrees
<i>l</i> (-)-Tryptophane (8)*	274-281	277 (8) 281-282 (9)	13.4-13.7	13.72	-32.0 to -33.5	-33.0 to -33.6 (10)
<i>dl</i> -Tryptophane (11)	280	282-283 (12)	13.6	13.72	0.0	
<i>d</i> (+)-Tryptophane (9)	280	281-282 (9)	13.6	13.72	+32.1	+32.45 (9)
<i>dl</i> -Indolelactic acid (12)	143	145 (12)	6.7	6.83	0.0	
<i>d</i> (-)-Indolelactic acid (12)	99	100-101 (12)	6.7	6.83	-5.3	-5.36 (12)
β -3-Indolepyruvic acid (13, 14)	205	211 (12)	6.9	6.89		
<i>l</i> (+)-Kynurenine sulfate† (4)‡	175 185	180 (4)‡	7.8-8.1	8.38	+10.1§	+10.7 +10.03 (4)‡

* The references which follow the names of compounds are to methods of preparation.

† In the free form, kynurenine is levorotatory ((4) and foot-note 1).

‡ See foot-note 1.

§ The concentration was 1.0 gm. per 100 cc. of solution in water; in all other instances the concentrations were 0.5 gm. per 100 cc. of aqueous solution.

Kjeldahl procedure. Optical rotations were read in a Schmidt-Haensch polariscope with an electric sodium lamp. Pertinent analytical data and comparisons with the literature are summarized in Table I.

Kynurenic acid, which is produced from several of these compounds, separates from the urine upon strong acidification. Kynurenine, tryptophane, and indole derivatives form sparingly soluble complexes with the mercuric sulfate reagent used in the Van Slyke procedure for determining total acetone bodies (16). Hence the following routines were devised to

determine kynurenic acid, "kynurenine," and total acetone bodies in each 24 hour sample of urine.

Kynurenic acid was precipitated by acidifying the urine to Congo red with sulfuric acid. After 24 hours in the refrigerator the precipitate was separated by centrifugation and washed by suspending it in 10 cc. of 5 per cent (by volume) sulfuric acid and recentrifuging. The supernatant fluid and washings were decanted into a 100 cc. volumetric flask. The kynurenic acid was redissolved, reprecipitated, and washed with water-saturated butyl alcohol to remove extraneous indole derivatives, essentially as directed elsewhere (6). The supernatant urine and washings were diluted to volume with water and reserved for estimating "kynurenine" and total acetone bodies.

"*Kynurenine*" refers collectively to all substances precipitable as the mercuric sulfate complex from urines previously freed of kynurenic acid, acetone, and acetoacetic acid; for convenience in making comparisons, the precipitates were calculated as kynurenine. For the determination, 20 cc. of the reserved supernatant urine and washings were mixed with 1 cc. of 50 per cent (by volume) sulfuric acid and boiled to expel the preformed acetone and the acetone produced by the decomposition of the acetoacetic acid. The solution was cooled, adjusted to 10 cc., and mixed with 20 cc. of 10 per cent mercuric sulfate in 5 per cent (by volume) sulfuric acid solution. After 48 hours, the precipitate which formed was filtered off on tared, sintered glass crucibles, washed with 10 cc. of 5 per cent sulfuric acid, then with 10 cc. of water, dried at 110°, and weighed. Similar precipitation of known weights of kynurenine with mercuric sulfate indicated that 1 mg. of the complex was equivalent to approximately 0.23 mg. of kynurenine. This factor was used in calculating "kynurenine."

Total acetone bodies were determined on 50 cc. of the reserved supernatant urine and washings, clarified as directed by Van Slyke (16) and made up to a volume of 250 cc. for filtration. 25 cc. of the filtrate were mixed, in a 500 cc. distilling flask, with 5 cc. of 17 N sulfuric acid and 100 cc. of water. The distilling flask was connected, through ground glass joints, to a condenser and a delivery tube extending nearly to the bottom of a 500 cc. Erlenmeyer flask which contained a mixture of 5 cc. of 17 N sulfuric acid and 17.5 cc. of 10 per cent mercuric sulfate in 4 N sulfuric acid solution. Preformed acetone and acetone produced by the decomposition of acetoacetic acid were distilled over until the volume of the solution in the distilling flask had been reduced by half. The residue was then cooled, 17.5 cc. of the 10 per cent mercuric sulfate reagent were added, and the mixture was set aside for 48 hours to allow the "kynurenine" to precipitate. The precipitate was filtered off and washed with 5 to 10 cc. of water. The filtrate and washings, which still contained β -hydroxybutyric acid, were

added to the distillate collected previously. Total acetone bodies were determined on the mixture by heating it to boiling, adding 5 cc. of 5 per cent potassium dichromate solution, and refluxing for $1\frac{1}{2}$ hours to oxidize the β -hydroxybutyric acid to acetone and to precipitate the acetone as the mercuric sulfate complex. Total acetone bodies were computed with the usual assumptions that 75 per cent was excreted as β -hydroxybutyric acid and that, upon oxidation, this product yielded 75 per cent of the acetone to which it was equivalent (16).

Analysis of urines containing acetone bodies alone gave essentially the same results with the modified procedure as with the usual routine. Addition of kynurenine acid and tryptophane, indolelactic acid, indolepyruvic acid, or kynurenine to such urines showed no appreciable interference.

The female rats used in the acetone body studies weighed 160 to 190 gm. each. They were housed in individual metabolism cages allowing collection of urine under oil and were fasted for 24 hours preceding each test period. Regenerated cellulose and water were available continuously. The compound tested was fed by stomach tube as a suspension in gum tragacanth, in doses of 2 cc. every 12 hours. Each dose contained 0.3 gm. of tryptophane (or its molecular equivalent of whatever other substance was tested), 0.0267 gm. of gum tragacanth, and 0.3 gm. of sodium butyrate or its equivalent of sodium chloride. The test material was dissolved in water containing sodium hydroxide equivalent to the ultimate sodium butyrate or sodium chloride content required. The powdered gum tragacanth was stirred in, and ample time was allowed for the mixture to become homogeneous. Butyric acid or hydrochloric acid exactly equivalent to the sodium hydroxide was then added, followed by enough water to dilute to appropriate volume. This yielded a smooth, permanent suspension which passed readily through the catheter used as a stomach tube.

Some of the compounds were fed for 3 days, some for only 2. Kynurenine was available as the sulfate; when this was used, enough additional sodium hydroxide was added to effect the liberation of the kynurenine. Control animals received suspensions prepared in the same way and containing equal concentrations of all of the components except the test material. The fasting was continued for 36 hours after the final feeding. The cages were rinsed and the urine was drawn off every 24 hours. Usually two tests were made on each animal. In the first, half of the rats served as the experimental subjects, the rest as controls; in the second, the regimens were reversed. When the same rats were used in successive tests, periods of at least 10 days on a stock diet of Purina dog chow intervened.

The experimental data are presented in condensed form in Table II. Equivalent weights of *l*(-)-tryptophane and *l*(-)-kynurenine produced approximately equal outputs of kynurenine acid. Indolepyruvic acid

yielded less, *dl*-tryptophane only a small amount, and the indolelactic acids and *d*(+)-tryptophane little or none.

TABLE II

Average Total Urinary Output of Kynurenic Acid, "Kynurenine," and Acetone Bodies in Female Rats Fed Tryptophane and Related Compounds, with or without Sodium Butyrate, after Preliminary 24 Hour Fast

No of rats*	Days of feeding†	Kynurenic acid	"Kynurenine"	Acetone bodies calculated as acetone	Substances fed in gum tragacanth suspension‡
		mg	mg	gm per sq m	
8	3	106	111	0 58	<i>l</i> (-)-Tryptophane + NaCl
8	3	14	27	0 35	NaCl
8	3	49	473	0 45	<i>dl</i> -Tryptophane + NaCl
8	3	35	43	0 34	NaCl
4	3	49	660	0 62	<i>d</i> (+)-Tryptophane + NaCl
4	3	76	87	0 18	NaCl
2	3			0 10	Ammonium chloride
2	3			0 13	None
24	3	166	175	1 30	<i>l</i> (-) Tryptophane + Na butyrate
24	3	37	34	4 35	Na butyrate
8	3	39	297§	0 92	<i>dl</i> -Tryptophane + Na butyrate
8	3	35	41	4 96	Na butyrate
6	3	29	589§	1 36	<i>d</i> (+)-Tryptophane + Na butyrate
6	3	31	87	4 71	Na butyrate
10	3			2 81	NH ₄ Cl + Na butyrate
10	3			4 80	Na butyrate
3	2	22	724§	2 29	<i>dl</i> -Indolelactic acid + Na butyrate
3	2	24	81	4 01	Na butyrate
4	2	26	797§	2 26	<i>d</i> (-)-Indolelactic acid + Na butyrate
4	2	88	262	0 91	Indolepyruvic acid + Na butyrate
4	2	104	113	0 68	<i>l</i> (-)-Kynurenine + Na butyrate
12	2	19	49	3 98	Na butyrate

* The rats weighed 160 to 190 gm each. Meeh's formula was used in calculating surface area

† An extra day of fasting was allowed to insure collection of all of the urine voided during the metabolism period

‡ Per day each rat received 0 053 gm of gum tragacanth and 0 6 gm of sodium butyrate or its equivalent of sodium chloride In addition, each experimental animal was fed 0 6 gm of tryptophane or its equivalent of other test material

§ These precipitates responded to the glyoxylic acid test See the text

Since the composition of the "kynurenine" precipitates was obscure, these were analyzed for total nitrogen. Estimation of amino nitrogen failed to give reproducible results and was abandoned, as a differential test, in favor of the Shaw and McFarlane quantitative adaptation of the glyoxylic acid

color reaction (17) for tryptophane. Mercuric sulfate precipitates obtained from pure solutions of tryptophane, kynurenine, and indolelactic acid showed nitrogen contents of 2.79, 2.70, and 1.23 per cent, respectively. Similar precipitates isolated after *l*(-), *dl*-, and *d*(+)-tryptophane and *l*(-)-kynurenine were fed contained 2.4 to 2.9 per cent of nitrogen; those obtained after indolepyruvic acid and *d*(-)-indolelactic acid were fed contained 1.9 and 1.4 per cent, respectively. Previous tests of the Shaw and McFarlane procedure had shown that kynurenine, indolepyruvic acid, and indole do not respond typically, but that skatole and indolepropionic acid produce colors qualitatively similar to the color formed with tryptophane.² The latter was found to be true also of indolelactic acid. Application of the method to pooled mercuric sulfate precipitates formed in the urines voided after *l*(-)-tryptophane, *l*(-)-kynurenine, or indolepyruvic acid was fed yielded no color; the "kynurenine" excreted after *dl*-tryptophane feeding produced a color equivalent to a content of 10 per cent of tryptophane; after *d*(+)-tryptophane feeding, to 33 per cent. The color produced by similar precipitates after *d*(-)-indolelactic acid feeding was compared with that developed in an indolelactic acid standard; apparently 88 per cent of the "kynurenine" was in this form. Unfortunately similar quantitative tests were not made after *dl*-indolelactic acid was fed. The colorimetric assays and the nitrogen data suggest that the substance precipitated from the urines of rats fed *l*(-)-tryptophane was chiefly kynurenine, that some kynurenine was probably produced from indolepyruvic acid, but that none was formed from *d*(-)-indolelactic acid. Subtraction of kynurenine equivalent to the estimated tryptophane content from the total "kynurenine" precipitated after *dl*- and *d*(+)-tryptophane were fed leaves a larger balance of "kynurenine" in each instance than was obtained after *l*(-)-tryptophane was fed. The precipitates probably contained *d*(+)-kynurenine which is formed from *d*(+)-tryptophane (15),¹ but cannot be converted into kynurenic acid (18), and hence must either be excreted as such or undergo further metabolism by some other route. Products resulting from intestinal putrefaction and subsequent detoxication may have been present, but probably in no greater amount than after *l*(-)-tryptophane was fed.

The average total acetone body excretion was slightly greater in the fasted rats fed tryptophane and sodium chloride than in their controls, but in rats fed sodium butyrate to augment the ketonuria, supplementation with tryptophane decreased the output markedly. Analysis showed that the acetone body precipitates from the urines of rats fed tryptophane contained an appreciable amount of nitrogen (an average of 0.084 per cent, as

² Buck, D. M., and Berg, C. P., unpublished data.

compared with 0.003 per cent for controls). (oprecipitation of nitrogenous products was therefore probably at least partly responsible for the slightly greater average weight of the mercuric sulfate-acetone complex produced in the urines of fasted rats fed tryptophane and sodium chloride than in the urines of their controls. On the other hand, the actual decrease in output of acetone bodies in the rats fed the compounds with sodium butyrate was probably slightly larger than the tabulated data indicate.

TABLE III
Deposition of Liver Glycogen in Rats Fed Tryptophane and Related Compounds after 48 Hour Fast

Substance fed*	Additional fasting or feeding period	No. of rats†	Minimum	Maximum	Average
	hrs.		per cent	per cent	per cent
<i>l</i> (-)-Tryptophane + NaCl	4	6	0.24	0.46	0.36
	8	6	0.11	0.67	0.30
	12	6	0.15	0.52	0.33
	20	4	0.20	0.46	0.35
<i>dl</i> -Tryptophane + NaCl	4	4	0.11	0.71	0.30
	12	4	0.11	0.45	0.23
<i>d</i> (+)-Tryptophane + NaCl	8	4	0.11	0.40	0.26
<i>dl</i> -Indolelactic acid + NaCl	8	4	0.15	0.74	0.41
Indolepyruvic acid + NaCl	8	4	0.18	0.60	0.36
<i>l</i> (-)-Kynurenine + NaCl	8	4	0.03	0.11	0.07
NaCl	4	6	0.12	0.56	0.26
	8	8	0.11	0.97	0.49
	12	8	0.37	0.77	0.49
	20	2	0.12	0.42	0.27

* Each rat received 0.026 gm. of gum tragacanth and 0.16 gm. of NaCl every 4 hours; each experimental animal was fed simultaneously also 0.3 gm. of tryptophane or its equivalent of other test material.

† The rats weighed 100 to 125 gm. each and were evenly divided as to sex.

Edson has observed that ammonium chloride stimulates the production of acetoacetic acid in liver slices from the well fed rat, but is without effect on liver slices from the fasted animal (19). It is of interest to note that administration of ammonium chloride to our rats in amounts molecularly equivalent to the tryptophane and other test substances had little effect in the animals which were simply fasted, but reduced appreciably the total acetone body output in the rats fed sodium butyrate. Correlation of this reduction with the greater reduction observed when tryptophane, indolepyruvic acid, or kynurenine was fed is complicated by uncertainty as to the relative rates of metabolism of these substances and the metabolic

paths followed. The lowering produced by the ingestion of indolepyruvic acid and of kynurenine was proportional to that produced by tryptophane in the same period; the reduction by the indolelactic acids was much smaller. The data obtained by Edson seem to show that *l*(-)-tryptophane lowers acetoacetic acid production in liver slices from fasted rats.

Substances which decrease the output of acetone bodies usually produce glycogen. To determine whether this was true of indolepyruvic acid, *l*(-)-kynurenine, and the isomers of tryptophane, these substances and *dl*-indolelactic acid were fed to rats subsequently examined for liver glycogen. Equal numbers of males and females, 100 to 125 gm. in weight, were removed from a stock diet of Purina dog chow and fasted for 48 hours before the compounds were fed. During the fasting and feeding periods they had continuous access to water and regenerated cellulose. Each substance tested was fed in a gum tragacanth suspension prepared with sodium hydroxide and hydrochloric acid as described in the acetone body studies. The experimental animals received 0.3 gm. of tryptophane or its equivalent every 4 hours; the controls were given all of the components of the suspension except the substance under investigation.

Groups of animals were killed for analysis by a blow on the head 4, 8, 12, or 20 hours after the initial feeding or the 48 hour fast. Their livers were removed at once and analyzed in their entirety for glycogen by the Good, Kramer, and Somogyi procedure (20); the glucose liberated by hydrolysis was determined by the Somogyi method (21).

The data are summarized in Table III. They do not favor the assumption that *l*(-)-, *dl*-, or *d*(+)-tryptophane, *l*(-)-kynurenine, *dl*-indolelactic acid, or indolepyruvic acid was glycogenic under the conditions employed. At present we have no explanation for this apparent discrepancy with data on other substances which similarly lower the acetone body output, but are also glycogenic.

SUMMARY

In rats fasted for 24 hours, then fed 0.6 gm. of *l*(-)-, *dl*-, or *d*(+)-tryptophane per day, the excretion of total acetone bodies appeared to be slightly greater than in control animals, but the small difference was probably due to contamination of the acetone-mercuric sulfate complex with traces of other compounds which contained nitrogen. In rats fed sodium butyrate to increase the ketonuria, similar administration of *l*(-)-, *dl*-, or *d*(+)-tryptophane reduced the acetone body output markedly. Proportionate decreases were produced by both *l*(-)-kynurenine and indolepyruvic acid, but the *d*(-)- and *dl*-indolelactic acids were much less effective. The feeding of molecularly equivalent amounts of ammonium chloride lowered the excretion of acetone bodies enough in the rats fed sodium butyrate to

suggest that production of ammonia and formation of urea from the other compounds tested may have been partly responsible for their effect in decreasing the acetone body output. However, since the decrease produced by indolepyruvic acid, which contains only indole nitrogen, was approximately equivalent to that induced by tryptophane, which contains both indole and amino nitrogen, and to the decrease induced by kynurenine, which contains two amino groups, other factors must also have been involved.

In rats fasted for 48 hours, then fed 0.3 gm. of *l*(-)-, *dl*-, or *d*(+)-tryptophane (or its equivalent of *dl*-indolelactic acid, indolepyruvic acid, or *l*(-)-kynurenine) at 4 hour intervals for periods of 4, 8, 12, or 20 hours, no evidence for storage of liver glycogen was obtained. This would seem to indicate that the effect of these substances upon the acetone body output in the rats fed sodium butyrate cannot be ascribed to their conversion into glucose or a glycogenic intermediate, unless acetone body formation or excretion can be affected by amounts too small to induce glycogen storage.

The yields of kynurenic acid and kynurenine from *l*(-)-tryptophane were proportionately the same as from *l*(-)-kynurenine, but did not account for more than a fourth of the total ingested. Proportionate depressions in acetone body output indicate that the metabolism of *l*(-)-kynurenine is not limited to its conversion to kynurenic acid; quite likely *l*(-)-kynurenine may be an intermediate also in the metabolism of the large portion of exogenous tryptophane which is not eliminated as kynurenic acid. Little kynurenic acid was produced from *dl*-tryptophane and apparently none from *d*(+)-tryptophane; the mercuric sulfate precipitates obtained after these were fed contained appreciable amounts of tryptophane, but probably consisted chiefly of *d*(+)-kynurenine. A little over half of the *d*(-)-indolelactic acid fed was excreted; the accompanying decrease in acetone body output suggests that the rest was metabolized, but the data do not warrant concluding that either kynurenic acid or appreciable kynurenine was produced. Indolepyruvic acid was converted to kynurenic acid, apparently more readily than previously noted in the rabbit, though less readily than *l*(-)-tryptophane; the high nitrogen content of the mercuric sulfate precipitate and its negative response to the test for tryptophane suggest that it was composed in part of indolepyruvic acid and in part of kynurenine.

BIBLIOGRAPHY

1. Borchers, R., and Berg, C. P., *Federation Proc.*, **1**, pt. 2, 102 (1942).
2. Rose, W. C., *Science*, **86**, 298 (1937).
3. Gordon, W. G., Kaufman, R. E., and Jackson, R. W., *J. Biol. Chem.*, **113**, 125 (1936).

4. Kotake, Y., and Iwao, J., *Z. physiol. Chem.*, **195**, 139 (1931).
5. Correll, J. T., Berg, C. P., and Cowan, D. W., *J. Biol. Chem.*, **123**, 151 (1938).
6. Berg, C. P., *J. Biol. Chem.*, **91**, 513 (1931).
7. Dakin, H. D., *J. Biol. Chem.*, **14**, 321 (1913).
8. Cox, G. J., and King, H., in Clarke, H. T., *et al.*, *Organic syntheses*, New York, **10**, 100 (1930).
9. Berg, C. P., *J. Biol. Chem.*, **100**, 79 (1933).
10. Berg, C. P., and Potgieter, M., *J. Biol. Chem.*, **94**, 661 (1931-32).
11. du Vigneaud, V., and Senlock, R. R., *J. Biol. Chem.*, **96**, 511 (1932).
12. Bauguess, L. C., and Berg, C. P., *J. Biol. Chem.*, **104**, 675 (1931).
13. Boyd, W. J., and Robson, W., *Biochem. J.*, **29**, 555 (1935).
14. Berg, C. P., Rose, W. C., and Marvel, C. S., *J. Biol. Chem.*, **85**, 219 (1929-30).
15. Kotake, Y., and Ito, N., *J. Biochem., Japan*, **25**, 71 (1937).
16. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
17. Shaw, J. L. D., and McFarlane, W. D., *J. Biol. Chem.*, **132**, 387 (1910).
18. Kotake, Y., and Ito, N., *J. Biochem., Japan*, **26**, 161 (1937).
19. Edson, N. L., *Biochem. J.*, **29**, 2082, 2498 (1935).
20. Good, C. A., Kramer, H., and Somogyi, M., *J. Biol. Chem.*, **100**, 485 (1933).
21. Somogyi, M., *J. Biol. Chem.*, **70**, 599 (1926).

THE OXIDATIVE CONVERSION OF CASEIN INTO PROTEIN FREE OF METHIONINE AND TRYPTOPHANE*

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It has been shown (1) that as a constituent of the diet of young rats methionine sulfoxide is capable of supporting growth to apparently the same extent as methionine. However, when a 2nd oxygen atom is added to the sulfur of methionine, the resulting sulfone seems to be entirely incapable of being metabolically reconverted into methionine, since no growth resulted when it was used as a dietary substitute for methionine (2). In view of this finding, and since the sulfone showed no evidence of toxicity, the possibility was considered of producing methionine-free protein by inactivating the amino acid within the protein molecule by oxidation to the sulfone level.

The experimental approach was based on three considerations: (a) oxidation of methionine to the sulfone (3) appears to involve the action of a per-acid (permolysidic acid), (b) proteins are soluble in formic acid, and (c) formic acid and hydrogen peroxide are capable of forming the per-acid performic acid (4). The work was undertaken on casein in view of its rôle in nutritional investigations. By subjecting casein dissolved in formic acid to the action of hydrogen peroxide, according to the procedure detailed in the experimental part, a modified casein has been obtained which seems to differ from normal casein chiefly in that it is devoid of methionine as well as tryptophane. The conclusion that these are the only "essential" (5) amino acids eliminated by the oxidation is based on biological evidence reported separately (6).

EXPERIMENTAL

Reagents—The reagents needed are casein, pulverized and passed through an 80 mesh sieve, 30 per cent hydrogen peroxide, water, methanol, 88 per cent formic acid, and concentrated ammonium hydroxide. The normality of the latter two reagents must be determined before use.

Preparation—In a jar equipped with a variable speed stirrer of monel metal 400 gm. of casein are gradually dissolved in 2400 cc. of formic acid, 400 cc. of hydrogen peroxide are rapidly added, and 2 or 3 minutes later

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stirring is stopped. After the peroxide has acted for 1 hour, during which time cooling coils are installed in the jar, 10 liters of water are added to the solution, and the stirrer is again put into action. This is immediately followed by ammonia. Optimal precipitation of the protein is obtained when the solution is neutralized to an acidity level of between pH 4 and 4.5, requiring neutralization of about 65 equivalent per cent of the formic acid used. Cool water is circulated and the solution rapidly stirred while the ammonia is added through a tube ending near the bottom of the jar (7); the temperature of the solution is not permitted to exceed 35°. Foaming is effectively combated by repeatedly spraying caprylic alcohol onto the solution. The protein precipitate is filtered and resuspended in 15 liters of water. After filtering and resuspending in water a second time, the precipitate is next suspended in 12 liters of methanol.¹ The suspension is left to settle overnight. This is followed by filtration and a second suspension in methanol overnight. The filtered material is now dried over calcium chloride, first in a desiccator and then in a vacuum drying oven at 70°. When the material approaches constant weight, it is thoroughly pulverized, to pass a 40 mesh sieve, and drying is continued until the loss during 4 hours at 70° and about 20 mm. of Hg does not exceed 0.1 per cent. For analytical and biological work the material was used in this state. The yield at this point corresponded to about 80 per cent of the weight of casein used.

The effectiveness of the washing method outlined was controlled by titrimetric determinations. Hydrogen peroxide and per-acid were determined iodometrically in filtrate and washings, and ammonium formate, resulting from the neutralization of the solvent, was titrated in the methanol washings as an acid by sodium methylate (8). The data obtained indicated that the final product is contaminated with less than 0.02 per cent of ammonium formate.

Analytical and Other Characteristics—Analytical results obtained on the product described and on its parent protein (vitamin-free casein, Smaco) are summarized in Table I. In addition to determinations of methionine, tryptophane, and cystine, amino acid analyses have been limited to threonine and serine because these compounds seem particularly susceptible to potential oxidative degradation influences (10, 11). When these two β -hydroxyamino acids were found intact, it appeared likely that no other types of amino acids were affected by the treatment. This view has in the meantime been confirmed, at least as far as the "essential" (5) amino acids are concerned, by biological evidence (6).

¹ Commercial methanol (99.8 per cent) was found definitely superior to acetone for the present drying purpose, with respect to filtrability and appearance of the resulting product as well as ease of removal.

No direct determination of the isoelectric point of the oxidized casein has been made. However, colorimetric estimations of the pH of all aqueous filtrates and washings showed consistently that the washings tended to be more acid than the filtrate from the neutralized solution and that the washings approached the value of pH 4.1. The conclusion seems justified, therefore, that the isoelectric point of the modified casein is definitely more acidic than that of casein (pH 4.6), being located at approximately pH 4.1.

TABLE I
Analytical Data on Oxidized Casein and Casein

All results are expressed as percentages of the protein.

	Oxidized casein		Casein	
Loss on drying (80°, vacuum, 43 hrs. (9)) ..	1.10	1 24	4 41	4 48
Total nitrogen (Kjeldahl)	14.17	14 23	14 04	14.16
Ammonia, after 4 hrs. hydrolysis*	1 71			
“ “ 22 “ “ †	1 85			
Threonine (10), after 13 hrs. hydrolysis†	3 67	3.71		
“ “ 22 “ “ †	3 69	3.70	3 52	3 56
Serine (11), after 13 hrs. hydrolysis†	4 84	4 87		
“ “ 22 “ “ †	4 57	4 81	4.08	4.42
Cystine (12), after 13 hrs. hydrolysis†	0 11	0.16	0.36	
Methionine (13), after 4 hrs. hydrolysis*	0 00			
“ “ 22 “ “ †	-0 01	+0 01		
“ (14), “ 22 “ “ †	-0 02	+0 03	2.6	2 7
Tryptophane (15), alkaline solution of protein	-0.01	+0 04	1 07	

* Method of rapid hydrolysis originated by Dr. T. F. Lavine: 2.5 gm. of protein, 4.5 cc. of concentrated HCl, and 1.1 cc. of H₂O are heated on the steam bath until solution occurs (about 15 minutes). The solution is refluxed for 4 hours, diluted, decolorized by boiling 1 minute with 500 mg. of activated carboraffin, and diluted with washing to 50 cc.

† 2.5 gm. of protein are refluxed with 50 cc. of 20 per cent HCl, once distilled to dryness *in vacuo*, decolorized, and diluted as in the foot-note above.

When the modified casein is hydrolyzed with hydrochloric acid, the absence (or mutilation) of tryptophane seems to find an expression in the complete failure of the transitory purplish colorations to appear which are so characteristic of the early stages of the hydrolysis of casein. Another distinguishing characteristic is that decolorized hydrolysates of the oxidized casein, unlike those of casein, turn yellow on being made alkaline and again become colorless when reacidified. On the other hand, an acid solution of the decolorized hydrolysate gradually develops a brown color on standing, a phenomenon which is likewise absent in the case of casein. Tests indicate that neither temperature nor the presence or absence of oxygen or light has a significant influence on this change. While the

nature of these properties is not understood, one may surmise that they are related to the oxidative alteration of tryptophane.

The writer is indebted to Dr. T. F. Lavine for the methionine determinations by his new method and for the determinations of ammonia, and to Mr. R. P. Homiller for help in the work of preparation.

SUMMARY

By treating it in formic acid solution with hydrogen peroxide casein has been converted into modified protein in which, according to chemical evidence, methionine and tryptophane are absent, and cystine has been partly destroyed, while threonine and serine (and presumably other amino acids) are present intact.

BIBLIOGRAPHY

1. Bennett, M. A., *Biochem. J.*, **33**, 1791 (1939).
2. Bennett, M. A., *J. Biol. Chem.*, **141**, 573 (1941).
3. Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, **140**, 131 (1941).
4. d'Ans, J., and Kneip, A., *Ber. chem. Ges.*, **48**, 1136 (1915).
5. Rose, W. C., *Science*, **86**, 298 (1937).
6. Bennett, M. A., and Toennies, G., *J. Biol. Chem.*, **145**, 671 (1942).
7. Cohn, E. J., and Hendry, J. L., in Clarke, H. T., *et al.*, *Organic syntheses*, New York, **10**, 16 (1930).
8. Lavine, T. F., and Toennies, G., *J. Biol. Chem.*, **101**, 727 (1933).
9. Snyder, R. S., and Hansen, H. C., *Ind. and Eng. Chem., Anal. Ed.*, **5**, 409 (1933).
10. Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138**, 91 (1941).
11. Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).
12. Shinohara, K., *J. Biol. Chem.*, **112**, 683 (1935-36).
13. Lavine, T. F., *Federation Proc.*, **1**, pt. 2, 120 (1942).
14. McCarthy, T. E., and Sullivan, M. X., *J. Biol. Chem.*, **141**, 871 (1941).
15. Shaw, J. L. D., and McFarlane, W. D., *Canad. J. Research, Sect. B*, **16**, 361 (1938).

A NUTRITIONAL ASSAY OF CASEIN MODIFIED BY THE ACTION OF HYDROGEN PEROXIDE AND FORMIC ACID*

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(Received for publication, July 2, 1942)

The preparation of a protein product derived from casein by an oxidative process, presumably involving oxidation of methionine groups to the sulfone stage, has been described by one of us (1). The chemical investigation of this product has been accompanied and supplemented by a study of its nutritional properties, with a view to ascertaining to what extent amino acids other than methionine may be chemically damaged by the process of preparation. There was visible evidence (1), during the process, of tryptophane being involved in the reaction, and among other amino acids threonine and serine were thought to be the most likely ones to be affected in view of their particular susceptibility to oxidative decomposition (2). The work to be described was begun on the basis of these considerations and subsequently yielded evidence indicating that among the "essential" amino acids (3) methionine and tryptophane are the only ones rendered nutritionally ineffective by the oxidation of casein by means of hydrogen peroxide in formic acid.

EXPERIMENTAL

Albino rats, Wistar strain, 25 to 28 days old were used as experimental animals. They were put on a diet of the following percentage composition: oxidized casein (1) (as the basal protein) varied, *L*-cystine 0.4, dextrin varied, sucrose 15, salt mixture 4 (Osborne and Mendel (4)), agar 2, and Mazola corn oil 30. The fat-soluble vitamins, carotene, viosterol, and α -tocopherol, were added to the corn oil in amounts that provided 200 U.S.P. units of vitamin A, 20 U.S.P. units of vitamin D, and 0.05 mg. of vitamin E per 4.5 gm. of basal food, while the water-soluble vitamins were given daily in sucrose solution by mouth from a pipette. The daily dose was 0.2 cc. per rat and contained the following:¹ thiamine hydrochloride, riboflavin, pyridoxine hydrochloride 40 γ each, calcium pantothenate 200 γ , nicotinic acid, *p*-aminobenzoic acid, and inositol 500 γ each, biotin methyl

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¹ Recommended by Dr. W. L. Sampson of Merck and Company, Inc., Rahway, New Jersey, to whom we express our appreciation.

ester 0.25 γ , ethanol 0.005 cc., and sucrose 90 mg. An aqueous solution of these compounds, with the exception of the sucrose, was prepared in twice the desired concentration, and divided into single portions large

TABLE I

Daily Basal Food Consumption per Rat in Gm. Averaged over 2 Day Period

Days	Unit A	Unit B	Unit C	Unit D	Unit E, control
6% casein + 0.4% cystine					
1-2	5.9	5.9	6.4	6.0	5.7
3-4	6.3	6.9	6.9	6.2	6.7
5-6	6.5	7.3	7.0	6.5	5.9
7-8	4.8	4.9	4.4	4.5	4.8
Average*...	5.8	6.1	6.0	5.8	5.7
6% oxidized casein + 0.4% cystine					
9-10	2.0	2.0	2.0	1.8	1.9
11-12	1.1	1.1	1.0	1.5	1.6
Average*...	1.5	1.5	1.5	1.6	1.7
6% oxidized casein + 0.4% cystine + supplements as follows					
	15 mg. <i>dl</i> -methionine, 13 mg. <i>l</i> -tryptophane, 79 mg. <i>dl</i> -threonine	15 mg. <i>dl</i> -methionine, 13 mg. <i>l</i> -tryptophane, 50 mg. glycine	15 mg. <i>dl</i> -methionine, 79 mg. <i>dl</i> -threonine, 5 mg. glycine	13 mg. <i>l</i> -tryptophane, 79 mg. <i>dl</i> -threonine, 8 mg. glycine	62 mg. glycine
13-14	1.2	0.8	0.7	1.6	0.9
15-16	1.8	2.4	1.0	1.6	1.3
17-18	3.0	2.8	1.9	1.7	1.6
19-20	3.9	3.3	1.7	2.0	1.4
21-22	3.2	2.8	0.2	1.8	1.5
23-24	3.7	3.9	1.4	1.9	1.5
25-26	3.1	3.4	2.1	1.5	1.7
27-28	3.3	3.9	2.0	1.8	2.0
29-30	3.5	3.7	2.0	2.0	1.7
31-32	3.4	3.6	1.4	1.6	1.0
33-34	3.1	4.1	1.5	1.6	0.9
35-36	3.3	3.0	1.0	2.1	0.9
Average*...	3.0	3.1	1.4	1.7	1.3

* Calculated from the single day figures.

enough for the daily requirement of all the experimental animals. These portions were kept at -10° and every day one of them was mixed with an equal volume of a 67 per cent sucrose solution. Choline chloride was

given separately, 20 mg. per rat per day in the first experiment and as 0.4 per cent of the basal diet in the second experiment.

The rats were kept in ordinary raised bottom cages with $\frac{1}{2}$ inch mesh screens and the temperature of the room was maintained at 29° (approximately $\pm 1^\circ$) by means of thermostatic control valves in the steam radiators. The temperature chosen seems to be nearly optimal for the well being of animals subsisting on a bare, heat-conducting surface. The animals were weighed every other day and the average weight of the groups

TABLE II

Daily Basal Food Consumption per Rat in Gm. Averaged over 2 Day Period

Days	Group A	Group B	Group C	Group D	Group E
10% oxidized casein + 0.4% cystine					
1-2	2.2	2.5	2.1	2.0	2.2
3-4	2.8	2.7	2.4	2.6	2.7
5-6	2.7	2.7	2.3	2.7	2.5
Average*...	2.6	2.5	2.2	2.4	2.5
10% oxidized casein + 0.4% cystine supplemented by					
	10 per cent casein + 0.4 per cent cystine + 0.1 per cent l-tryptophane	0.2 per cent l-tryptophane + 0.34 per cent dl-methionine	Unsupplemented	0.34 per cent dl-methionine	0.2 per cent l-tryptophane
7-8	6.2	4.9	1.9	2.2	2.0
9-10	5.8	4.6	2.3	2.4	2.0
11-12	7.0	5.2	2.1	2.4	2.0
13-14	6.2	5.2	2.1	2.1	1.8
15-16	7.2	5.8	2.1	1.6	1.8
17-18	6.6	5.0	1.9	1.5	1.8
Average*...	6.4	5.1	2.1	2.0	1.9

* Calculated from the single day figures.

plotted. The approximate food consumption was determined by weighing daily the food given each group and the residual food, the difference being divided by the number of animals in each group. These figures, combined into 2 day averages, are listed in Tables I and II.

Investigations of Amino Acids Destroyed in Oxidation of Casein—In this experiment seven male rats, litter mates, 25 days old, were divided into five units. Units A and E contained two rats each, Units B, C, and D one rat each. After 6 days on the normal colony diet all units were put on a 6 per cent casein (vitamin-free, Smaco) diet for 8 days to test the growth

response of the rats to the synthetic vitamin ration. The casein of the basal diet was then replaced by 6 per cent of oxidized casein for a period of 4 days, at the end of which the amino acid supplements plus choline were given daily mixed in butter. The diet of Unit A was supplemented daily for 24 days with 15 mg. of *dl*-methionine, 13 mg. of *l*-tryptophane, and 79 mg. of *dl*-threonine; that of Unit B with 15 mg. of *dl*-methionine, 13 mg. of *l*-tryptophane, and 50 mg. of glycine; that of Unit C with 15 mg. of *dl*-methionine, 79 mg. of *dl*-threonine, and 5 mg. of glycine; that of Unit D with 13 mg. of *l*-tryptophane, 79 mg. of *dl*-threonine, and 8 mg. of glycine; that of Unit E with 62 mg. of glycine. 79 mg. of threonine were fed to take care of the growth requirements of serine as well as of threonine,

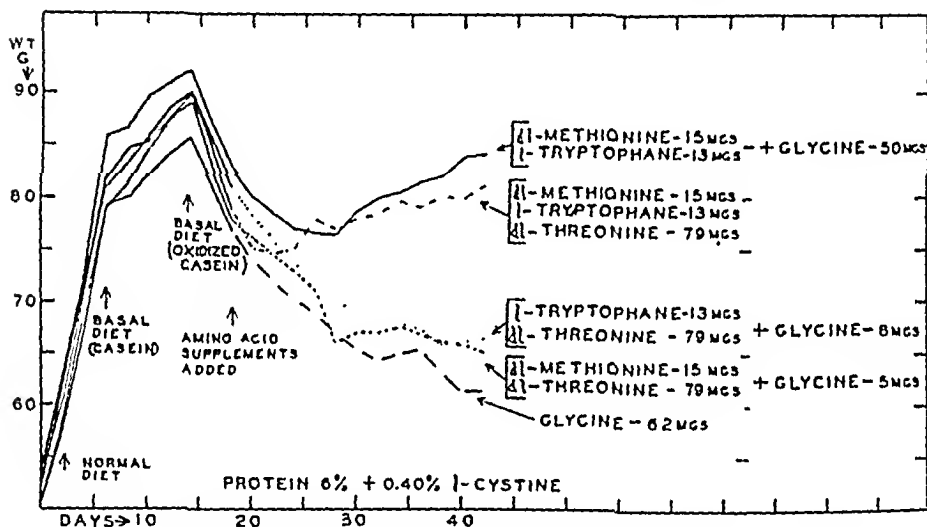


Fig. 1. Investigation of the amino acids destroyed in the oxidation of casein

according to the theory that threonine may be the source of serine in protein synthesis if only threonine is supplied. Glycine was added to supply an equivalent amount of nitrogen in each diet.

The rats of Units A and B showed an increase of 2.6 and 1.6 per cent respectively of the initial weight at which the supplements started. The rats of the other three units, C, D, and E, however, fell 16.5, 18.0, and 20.0 per cent below the initial weight (Fig. 1).

The basal food consumption, *ad libitum*, for the rats in Units A and B was about twice that of the rats in the units that showed no growth (Table I).

These results indicate that the oxidized casein is lacking in methionine and tryptophane but give no evidence of a threonine deficiency.

Nutritional Comparison of Casein with Adequately Supplemented Oxidized Casein—In order to obtain a more vigorous growth response for the purposes of this comparison the protein content of the diet was raised to 10 per cent at the expense of a corresponding amount of dextrin, the other components remaining essentially the same. The casein-containing diet was enriched by 0.10 per cent of tryptophane because of the recent demonstration (5)

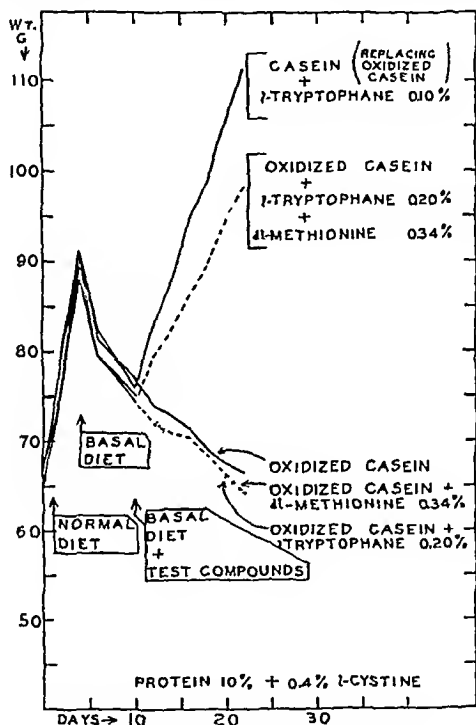


FIG. 2. Nutritional comparison of casein with adequately supplemented oxidized casein.

that the tryptophane content of casein is only approximately one-half of that commonly assumed previously. The amounts of amino acids used to supplement the oxidized casein were based on the amounts presumably present in casein, and the tryptophane supplement was adjusted accordingly. All supplements, including choline (0.4 per cent), were incorporated in the diet in this experiment, while the water-soluble vitamins were given as before. The choline chloride was homogeneously dispersed in the oil

component of the diet with the aid of 13 cc. of alcohol and 10 cc. of water per kilo of oil.

Twenty-one male rats from six litters, 26 to 28 days old, were divided into five groups. Groups A and B consisted of six rats each, Groups C, D, and E of three animals each. After 4 days on a normal diet, all groups were put on a diet containing 10 per cent of oxidized casein for 6 additional days. The diet of Group A was then changed to 10 per cent casein (vitamin-free, Smaco, supplemented with 0.10 per cent of *l*-tryptophane), while the basal protein in the diet of the other groups remained the same, 10 per cent of oxidized casein. The diet of Group B was supplemented with 0.20 per cent of *l*-tryptophane and 0.34 per cent of *dl*-methionine; that of Group C remained unsupplemented; that of Group D was supplemented with 0.34 per cent of *dl*-methionine; and that of Group E with 0.20 per cent of *l*-tryptophane.

During the 12 days on the supplemented diets the rats of Group A gained 47 ± 7 per cent (average deviation) and those of Group B 31 ± 5 per cent, while those of Groups C, D, and E showed losses of 13.3 ± 1.1 , 13.5 ± 0.8 , and 15.5 ± 0.5 per cent respectively (Fig. 2).

Here, just as in the first experiment, the adequacy of the diet is strongly reflected in the food consumption figures (Table II).

DISCUSSION

The experiments reported show that the growth-promoting properties of the casein (supplemented with cystine) have been destroyed by its conversion to oxidized casein. This is thought to be due, in part, to conversion of methionine in the protein molecule to the sulfone form (1) which has been found to be incapable of replacing methionine in the diet (6). However, it is of considerable interest in this connection that we found in another experiment, to be reported in detail later, positive growth on a choline-free diet containing 15 per cent of oxidized casein, supplemented with 0.36 per cent of *dl*-homocystine or *dl*-homocysteine, while supplementation with 0.16 per cent of *l*-cystine remained without effect. The implication of these observations that the methyl group of methionine sulfone may be utilized in the conversion of homocystine to methionine will be subjected to further investigation.

The present data further show, in agreement with the results of chemical analysis (1), that the oxidized casein lacks tryptophane but not threonine.

The fully supplemented oxidized casein seems to be a nutritionally adequate combination, since all of the "essential" amino acids (3) are present, although it is not as efficient in growth promotion as casein.

SUMMARY

Growth experiments carried out on albino rats have shown that in the oxidation of casein by hydrogen peroxide in formic acid only methionine

and tryptophane, among the essential amino acids, have been deprived of their growth-supporting properties.

Fully supplemented oxidized casein seems to be a nutritionally adequate combination, although it is not as efficient in growth promotion as casein.

BIBLIOGRAPHY

1. Toennies, G., *J. Biol. Chem.*, **145**, 667 (1942).
2. Shinn, L. A., and Nicolet, B. H., *J. Biol. Chem.*, **138**, 91 (1941). Nicolet, B. H., and Shinn, L. A., *J. Biol. Chem.*, **139**, 687 (1941).
3. Rose, W. C., *Science*, **86**, 298 (1937).
4. Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, **37**, 572 (1919).
5. Shaw, J. L. D., and McFarlane, W. D., *Canad. J. Research, Sect. B*, **16**, 361 (1938).
6. Bennett, M. A., *J. Biol. Chem.*, **141**, 573 (1941).

THE METABOLISM OF VALINE IN PHLORHIZIN GLYCOSURIA*

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Since the discovery of valine as a biological entity (1) little information has been accumulated concerning its metabolic significance. From the anabolic point of view it has been shown to be an indispensable dietary constituent (2). However, knowledge of its catabolic fate hinges to a large extent upon whether or not it possesses glyconeogenic properties. Experiments conducted by Dakin (3) upon phlorhizinized dogs indicated that glucose is not formed from valine in significant amounts; nevertheless, the excretion of acetoacetic acid decreased following its administration. The fact that it is not ketogenic had been shown earlier by Embden, Salomon, and Schmidt (4), who found no additional acetone bodies after perfusion of the liver with a solution containing the amino acid. From tissue slice studies, Cohen (5) obtained further evidence in support of the antiketogenic rôle of the compound, and in phlorhizinized animals Ringer, Frankel, and Jonas (6) found that isobutyric acid, a possible intermediate in valine catabolism, contributes 3 of its carbon atoms to the formation of glucose.

The apparent antiketogenic properties of valine and the fact that isobutyric acid is a glucose former are difficult to reconcile with the reported failure of the amino acid to yield sugar in the diabetic animal. The situation is further complicated by the recent findings of Butts and Sinnhuber (7) to the effect that small increases in liver glycogen, accompanied by significant decreases in ketonuria, follow the administration of *dl*-valine to fasting rats. Consequently, in an effort to obtain further information regarding the metabolism of valine in phlorhizin glycosuria, experiments were undertaken with *l*(+)-, *d*(-)-, and *dl*-valine, and with sodium α -ketoisovalerate. The procedure and the findings are outlined below.

EXPERIMENTAL

Healthy female dogs which had borne pups were used as the experimental subjects. Food was withdrawn from each animal 2 days before the first injection of phlorhizin, and fasting was continued throughout the experiment except for the administration of the compound under investigation.

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Water was furnished *ad libitum*. The phlorhizin was purified as directed by Denel and Chambers (8). Each dog received daily subcutaneous injections of 0.154 gm. per kilo of body weight. The drug was ground and suspended in 10 ml. of olive oil as recommended by Coolen (9). The urines were divided into 24 hour samples by catheterization, and were preserved under toluene until analyzed.

Total nitrogen was determined by the Scales and Harrison (10) modification of the Kjeldahl method, acetone bodies by the procedure of Van Slyke (11) with the factor proposed by Blunden *et al.* (12), and glucose according to the technique of Sumner (13). In the glucose estimations, the color intensity was measured by means of a photoelectric colorimeter.¹ In order to exclude the possibility that the results may have been influenced by renal retention, daily blood samples were analyzed for non-protein nitrogen in all but the first three experiments. For this purpose, the Koch-McMeekin procedure as described by Hoffman (14) was employed.

The dextro- and levorotatory enantiomorphs of valine were prepared by resolution of the racemic product according to the method of Fischer (15). Purity of the compounds was checked by analysis, and by determinations of their specific rotations as indicated in the accompanying tabulation.

		N	Specific rotation (aqueous solution)
l(+)-Valine.	Calculated.	11.96	$[\alpha]_D^{25} = +6.42^\circ$ (cf. (15))
	Found (three preparations).	12.06	$[\alpha]_D^{25} = +6.27^\circ$
		12.02	$[\alpha]_D^{25} = +6.12^\circ$
		12.06	$[\alpha]_D^{25} = +6.39^\circ$
d(-)-Valine.	Calculated.	11.96	$[\alpha]_D^{25} = -6.06^\circ$ (cf. (16))
	Found.	12.01	$[\alpha]_D^{25} = -6.05^\circ$
dl-Valine.	Calculated.	11.96	
	Found.	12.06	

The synthesis of sodium α -ketoisovalerate was accomplished by two different procedures. The method described by Bouveault and Locquin (17) was first employed, whereby ethyl α -ketoisovalerate was prepared. This was saponified by dissolving 45 gm. in 100 ml. of 99.5 per cent ethyl alcohol and treating with an alcoholic solution of 12.5 gm. of sodium hydroxide. A white, crystalline precipitate appeared immediately. After the mixture was cooled overnight at 5° , the salt was filtered off and recrystallized from water and ethyl alcohol to yield 33 gm. of sodium α -ketoisovalerate.

$C_5H_7O_3Na$.	Calculated.	C 43.48, H 5.11, Na 16.65
	Found.	" 43.15, " 5.48, " 16.59

The second method involved the hydrolysis of benzoyl- α -amino- β -methylcrotonic acid azlactone according to the procedure of Ramage and

¹ We have found this method to be thoroughly reliable when all conditions are rigidly controlled, and the period of heating is maintained for *exactly* 5 minutes.

Simonsen (18). 25 gm. of the resulting keto acid were dissolved in 100 ml. of 99.5 per cent ethyl alcohol and treated with a hot, saturated alcoholic solution of sodium hydroxide until neutral to moist litmus. The mixture was cooled overnight at 5° and filtered. The precipitate was recrystallized from water and alcohol to yield 27 gm. of sodium α -ketoisovalerate.

$C_5H_7O_3Na$. Calculated. C 43.48, H 5.11, Na 16.65
Found. " 43.67, " 5.23, " 16.67

The compounds were administered after the establishment of relatively constant G:N ratios, each for 2 to 3 days. This condition usually prevailed 4 or 5 days after the first injection of phlorhizin. It is important to the success of experiments with phlorhizinized dogs that all conditions be standardized as fully as possible. A fixed routine should be maintained from day to day and from dog to dog. Establishment of reasonably constant G:N ratios is imperative. However, once a basic value has been attained, the substance to be tested should be administered without further delay. Despite purification of phlorhizin, prolonged administration frequently results in kidney damage and nitrogen retention.

In our earlier experiments, valine was administered by stomach tube. This proved to be unsatisfactory because of the frequency with which vomiting occurred. In all later tests the compounds were injected intraperitoneally. This method proved to be far superior and made possible more easily duplicable results.

The extra glucose output was calculated by subtracting the average of the G:N ratios of the 1st, 2nd, and 4th days shown in Tables I to III from the G:N ratio (corrected for exogenous nitrogen) of the 3rd day and multiplying the difference by the endogenous nitrogen of the 3rd day. The theoretical or expected output was calculated upon the assumption that 1 mole of valine, or of sodium α -ketoisovalerate, is equivalent to 0.5 mole of glucose.

The results of the investigation are summarized in Tables I to III. In Table I are presented the findings in four experiments with *l*(+)-valine. In every case extra glucose was formed, and with a single exception 3 of the 5 carbon atoms were involved in the glycconeogenesis. No explanation is available for the low yield (46 per cent of the theory) in Dog 10.

In Table II are recorded the results of one experiment with *d*(-)-valine, and one with *dl*-valine. In each case, approximately theoretical yields of extra glucose were observed. This was quite unexpected, inasmuch as *d*(-)-valine, at least in the rat, is not interchangeable with the natural isomer for purposes of growth (19).

According to the generally accepted concept of amino acid catabolism, the keto acid is the first product of oxidative deamination. Table III

contains the results of two experiments wherein the sodium salt of α -ketoisovaleric acid was administered to phlorhizinized dogs. Again, glucose was formed in amounts equivalent to 3 carbon atoms.

In general the acetone excretion is consistent with the contention of other investigators that valine possesses antiketogenic properties. Two apparent exceptions are to be noted. Dog 18 showed a rise in the output of acetone on the day of valine administration. Dog 30 manifested a

TABLE I
Fate of l(+)-Valine in Phlorhizin Glycosuria

Dog No.	Weight	Date	Blood non-protein N	Urine				Extra glucose			l(+)-Valine administered
				Glucose	Total N	G:N	Acetone*	Calculated	Found	Conversion	
	kg.		mg. per cent	gm.	gm.		gm.	gm.	gm.	per cent	
10	7.0	Dec. 1		22.95	7.55	3.01	1.01				
		" 2		25.50	8.14	3.14	0.91				
		" 3		21.30	6.87	3.02†	0.75	9.16	4.19	46	11.94 gm., stomach tube
		" 4		21.30	6.50	3.28	0.75				
12	9.1	" 7		29.00	7.95	3.65	0.54				
		" 8		20.00	5.90	3.39	0.80				
		" 9		22.75	5.30	5.90†	0.33	9.21	9.07	98	12.0 gm., stomach tube
		" 10		13.50	3.75	3.61	0.45				
18	5.1	Jan. 30		16.75	5.55	3.02	2.22				
		" 31		13.50	4.65	2.90	0.75				
		Feb. 1		19.50	5.61	4.31†	1.39	6.91	6.03	87	9.0 gm., stomach tube
21	8.9	" 2		13.50	4.48	3.01	0.95				
		" 20	43	28.00†	8.62	3.25	0.87				
		" 21	41	27.00	8.24	3.28	3.08				
		" 22	39	31.50	8.69	4.21†	2.32	7.67	7.41	96	10.0 gm. in 275 ml. water injected intraperitoneally
		" 23	41	22.00	7.05	3.12	2.32				

* Total "acetone bodies" expressed as acetone.

† Corrected for the nitrogen content of the valine.

‡ Slight contamination of the urine with feces occurred on this day.

similar, though less pronounced, response to the injection of sodium α -ketoisovalerate. Probably both occurrences were fortuitous, and not subject to logical explanation. It is the usual experience in phlorhizin glycosuria that the excretion of acetone is more variable than that of other urinary components. A factor which doubtless contributes to this irregularity is the activity of the animal, and the resulting change in rate of pulmonary excretion. In any event, no evidence is afforded by the data

herein reported that valine, or its keto analogue, yields acetone in metabolism.

TABLE II
Fate of d(-)- and dl-Valine in Phlorhizin Glycosuria

Dog No.	Weight	Date	Blood non-protein N	Urine				Extra glucose			10.0 gm valine injected intraperitoneally
				Glucose	Total N	G:N	Acetone*	Calculated	Found	Conversion	
	kg.		mg. per cent	gm.	gm.		gm.	gm.	gm.	per cent	
28	12.0	Apr. 20	35	35.50	10.00	3.55	0.95				d(-)-Valine in 210 ml. water
		" 21	34	34.50	9.72	3.55	1.42				
		" 22	30	40.20	9.06	5.12†	0.84	7.67	8.48	110	
		" 23	69	34.00	6.78	5.02	0.60				
37	6.3	May 21	25	23.50	8.13	2.89	1.24				dl-Valine in 175 ml. water
		" 22	45	17.38	6.25	2.78	0.61				
		" 23		15.50	4.28	5.04†	0.27	7.67	7.27	95	
		" 24	31	15.00	6.35	2.36	1.05				

* Total "acetone bodies" expressed as acetone.

† Corrected for the nitrogen content of the valine.

TABLE III
Fate of Sodium α -Ketoisovalerate in Phlorhizin Glycosuria

Dog No.	Weight	Date	Blood non-protein N	Urine				Extra glucose		
				Glucose	Total N	G:N	Acetone*	Calculated	Found	Conversion
	kg.		mg. per cent	gm.	gm.		gm.	gm.	gm.	per cent
29	6.8	Apr. 28	38	19.75	6.13	3.22	0.68			
		" 29	37	20.50	6.08	3.37	0.80			
		" 30†	27	24.75	5.18	4.78	0.72	7.83	7.94	101
		May 1	32	15.75	4.99	3.16	2.12			
30	8.0	Apr. 28	32	21.25	6.30	3.38	1.92			
		" 29	32	19.75	6.42	3.08	2.02			
		" 30†	27	22.60	4.50	5.02	2.42	7.83	8.05†	103
		May 1		Animal died						

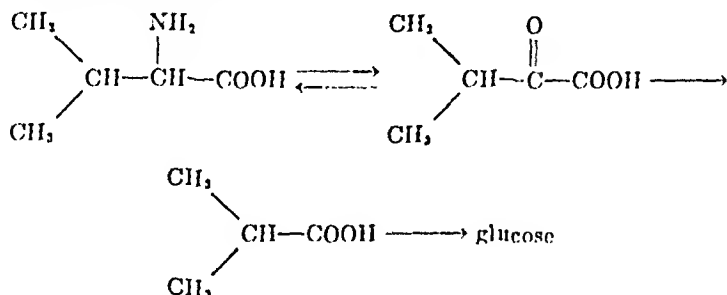
* Total "acetone bodies" expressed as acetone.

† 12.0 gm. of sodium ketoisovalerate in 200 ml. of water were injected intraperitoneally.

† The extra glucose in this experiment is calculated on the basis of the rise in G:N ratio above the average of the 2 preceding days.

The demonstration that valine and its α -keto derivative contribute 3 of their carbon atoms to the formation of glucose and the fact that iso-

butyric acid is a sugar former (6) indicate quite strongly that the amino acid in question is catabolized in the diabetic organism as represented by the accompanying formulas.



Of these reactions, the first is reversible, at least in the rat.² Further information is needed regarding the chemical mechanism involved in the conversion of isobutyric acid to glucose.

SUMMARY

In the phlorhizinized dog, *l*(+)-valine, *d*(-)-valine, *dl*-valine, and sodium α -ketoisovalerate contribute 3 of their 5 carbon atoms to the formation of glucose. The bearing of these facts upon the catabolism of valine is discussed.

BIBLIOGRAPHY

1. von Garup-Besanez, E., *Ann. Chem.*, **98**, 1 (1856).
2. Rose, W. C., and Eppstein, S. H., *J. Biol. Chem.*, **127**, 677 (1939).
3. Dakin, H. D., *J. Biol. Chem.*, **14**, 321 (1913).
4. Embden, G., Salomon, H., and Schmidt, F., *Beitr. chem. Physiol. u. Path.*, **8**, 129 (1906).
5. Cohen, P. P., *J. Biol. Chem.*, **119**, 333 (1937).
6. Ringer, A. I., Frankel, E. M., and Jonas, L., *J. Biol. Chem.*, **14**, 525 (1913).
7. Butts, J. S., and Sinnhuber, R. O., *J. Biol. Chem.*, **139**, 963 (1941).
8. Deuel, H. J., Jr., and Chambers, W. H., *J. Biol. Chem.*, **65**, 7 (1925).
9. Coolen, *Arch. pharmacod.*, **1**, 267 (1895).
10. Scales, F. M., and Harrison, A. P., *J. Ind. and Eng. Chem.*, **12**, 350 (1920).
11. Van Slyke, D. D., *J. Biol. Chem.*, **32**, 455 (1917).
12. Blunden, H., Hallman, L. F., Morehouse, M. G., and Deuel, H. J., Jr., *J. Biol. Chem.*, **135**, 757 (1940).
13. Sumner, J. B., *J. Biol. Chem.*, **65**, 393 (1925).
14. Hoffman, W. S., *Photometric clinical chemistry*, New York (1941).
15. Fischer, E., *Ber. chem. Ges.*, **39**, 2320 (1906).
16. Ehrlich, F., and Wendel, H., *Biochem. Z.*, **8**, 399 (1908).
17. Bouveault, L., and Locquin, R., *Bull. Soc. chim.*, **31**, 1142 (1904).
18. Ramage, G. R., and Simonsen, J. L., *J. Chem. Soc.*, 532 (1935).
19. Rose, W. C., *Physiol. Rev.*, **18**, 109 (1938).

² Rose, W. C., Johnson, J. E., and Eppstein, S. H., unpublished data.

OBSERVATIONS ON PROTEINASE IN BRAIN*

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For purely practical reasons, brain tissue is regarded by meat-packing technologists as prone to particularly rapid breakdown and spoilage. The presence of active autolytic agents in this tissue is thus to be expected. Yet previous investigators, while reporting the presence of a variety of hydrolytic enzymes, have not demonstrated the presence of any of them in remarkably large quantities. Nor has the autolysis of brain tissue, as judged by protein breakdown, appeared to be especially rapid or far reaching (1-5).

Proof of the presence of various types of proteolytic enzymes has been cited by these and other authors.

The presence of a nuclease in brain was reported by Craifaleanu (2); gelatinase, nuclease, and peptidase, by Takasaka (5); and a great variety of enzymes, including the foregoing plus trypsin, was noted by Slowtsoff (6). Edlbacher, Goldschmidt, and Schläppi (7) have demonstrated weak catheptic, but practically no tryptic, activity in brain. In general, slight proteolytic action has been commonly attributed to brain, although Blum, Jakovtshuk, and Jarmoschkewitsch (8) state that, of the various proteolytic enzymes, they found only a dipeptidase in appreciable amounts.

With regard to the effect of its proteolytic enzymes on the brain itself, Levene and Stookey (1) concluded, as early as 1903, that nerve tissue does not differ qualitatively from muscle, spleen, or liver in proteolytic activity, which (they noted) was favored by the addition of acid. Later Gibson, Umbreit, and Bradley (3) stated that brain tissues autolyze like other tissues, except on a smaller scale.

More recent experience with other tissues has indicated that new methods of measuring protein digestion accent the initial stages of the breakdown better than do the older methods, based on an increase in total amino nitrogen. In this study proteolysis due to the enzymes in brain has been followed by measuring the tyrosine liberated thereby from the proteins. The method was proposed by Hertzman and Bradley (9) for

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Enzyme Research Laboratory Contribution No. 80.

tracing autolysis, and has been developed by Anson (10) into a general procedure for the quantitative determination of proteinases. When the ability of the finely divided tissue to digest hemoglobin was determined by this method, calf brain was found to contain approximately 6 times as much proteinase as muscle. As the protein content of brain is roughly half that of muscle, the enzyme concentration on a protein basis is thus about 12 times that of muscle, or two-fifths that of spleen (11). The brain proteinase differs in no observed respect from the cathepsin found in liver, spleen, and muscle. A partially purified enzyme preparation, as well as fresh brain suspensions, digested hemoglobin at pH 3.5 without being activated or inhibited by cysteine or iodoacetamide. It was also observed that fresh brain tissue gave no evidence of digesting hemoglobin at pH 7.5, and the presence of a trypsin-like enzyme is therefore unlikely.

One or more peptide-splitting enzymes were found in fresh brain tissue. *dl*-Leucylglycine and *dl*-leucylglycylglycine were split at approximately the same rate by calf brain as by beef muscle, and glycyl-*l*-leucine and glycylglycine were split more rapidly by brain than by muscle, whereas with *dl*-alanylglycine the reverse is probably true. Extraction of fresh brain tissue with cold acetone and ether does not greatly affect the peptidase activity, except for the splitting of the glycyl peptides which is considerably reduced. Abderhalden and Caesar (12) have also reported the presence of peptidases in brain capable of splitting the above peptides.

Similar fresh and acetone-ether-extracted samples of brain tissue did not hydrolyze benzyl butyrate. Butyric acid was not found in significant amounts in a steam distillate from 5 gm. of brain which had been suspended in 45 ml. of H₂O and incubated with 0.5 ml. of benzyl butyrate for 5 hours at 40° (13). Since this test is apparently a very delicate one for the lipase of the pancreas and the esterase of liver and muscle, it is unlikely that esterases of this type are present in appreciable quantities, although there is evidence of the presence of these enzymes in brain presented in the literature (5, 6, 14).

EXPERIMENTAL

The surface layer of membranes and blood vessels was removed as well as possible from the brains of calves that had been slaughtered 2 to 3 hours previously. The brain tissue was next emulsified in 10 volumes of water in a small, high speed mechanical mixer with sharp blades. Portions of this water suspension were assayed for catheptic activity by mixing with buffered hemoglobin solution (pH 3.5) and determining the tyrosine liberated after 10 minutes incubation at 38° as described by Anson (10).

In experiments on the rate of autolysis the same suspension of brain tissue in water was preserved with toluene. Samples were periodically

withdrawn and the protein precipitated by the addition of trichloroacetic acid. 5 ml. of the filtrate (equivalent to 166 mg. of the original tissue) were used as before for the estimation of tyrosine. When the autolyses were carried out at pH 3.5 to 4.0 the pH of the brain suspension was adjusted by the careful addition of N HCl. Similar autolysis experiments were carried out with calf skeletal muscle. The results of a typical set of experiments are shown in Fig. 1.

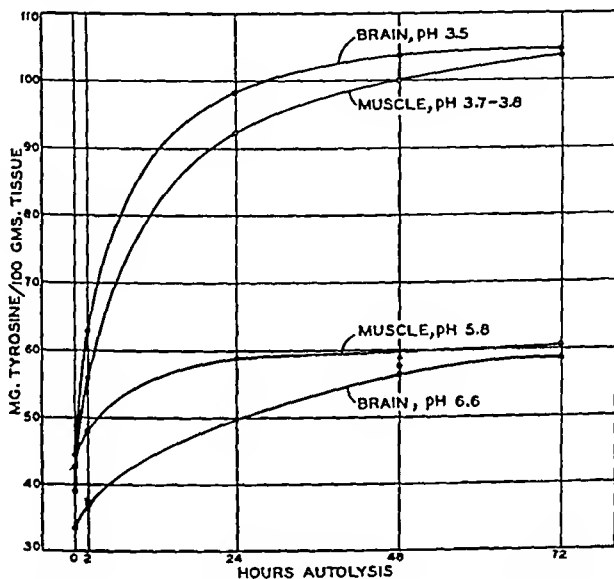


FIG. 1. Comparison of autolysis in brain and muscle as indicated by increase in soluble tyrosine.

For the preparation of cathepsin the fresh brain was first extracted with cold acetone and ether which served to remove water and practically all of the lipids. 60 gm. of the fresh brain were chopped finely with a knife and extracted twice (about 10 minutes each time) with 100 ml. portions of cold acetone. The tissue was filtered out and chopped again after each extraction. It was then similarly extracted twice (15 minutes) with 100 ml. portions of acetone-ether (1:1). It was finally extracted with two 100 ml. portions of cold ether (20 minutes) and left at 5° overnight in 50 ml. of ether. The tissue was then removed from the ether and dried in air at room temperature. After drying it was ground in a mortar and sieved. Approximately 90 per cent of the catheptic activity found in the original

tissue was retained in the dried material. Longer treatment with acetone and ether diminished the amount of recoverable enzyme.

The cathepsin was extracted from the dried tissue by suspending it in 20 volumes of 0.2 M acetic acid (pH of suspension, 3.7) for 1 hour at 7°. The undissolved material was filtered off and the protein in the filtrate precipitated by 0.8 saturation with ammonium sulfate. The precipitate was suspended in 0.2 M acetic acid and dialyzed overnight against cold distilled water. The insoluble material remaining after dialysis was removed by filtration. A water-clear filtrate containing 0.063 mg. of protein nitrogen and possessing an activity of 2.5×10^{-4} cathepsin unit per ml. was obtained. The specific activity of this preparation was 4×10^{-3} unit or approximately 65 times that of fresh brain. In the process of purification

TABLE I
Properties of Brain Proteinase

Enzyme preparation	Cathepsin units per ml. $\times 10^4$				
	pH 2.4	pH 3.5	pH 4.3	pH 7.5	pH 3.5*
Suspension of fresh calf brain, 1:10		0.75		No activity in 1 hr.	0.75
Solution of protein from calf brain, 1 ml. = 0.061 mg. protein N	1.8	2.5	1.0		2.4

1.0 ml. of 0.1 M cysteine hydrochloride neutralized and mixed with hemoglobin before the enzyme was added had no effect in either experiment. In the control experiment an equal amount was added just before precipitation with trichloroacetic acid.

* After alkaline iodoacetamide treatment. 0.5 ml. of 0.02 M iodoacetamide was mixed with 1 ml. of enzyme solution (pH 7 to 8) 1 hour before assay.

about 85 per cent of the proteolytic activity originally present in the brain tissue was lost. A large part of this loss was due to the handling of small quantities of material. The properties of the purified enzyme are shown in Table I.

The preparations of partly purified enzyme described above were also assayed by the method of Anson, with the use of hemoglobin as a substrate. The quantity of enzyme has been expressed in hemoglobin units (Anson (10)), with the aid of a curve correlating color values and units that was made from data obtained with hemoglobin and the purified brain cathepsin. The specific activity is the number of units per mg. of protein nitrogen. Protein nitrogens were determined by the micro-Kjeldahl procedure.

The peptidase activity was determined by a modified formol titration of aliquots of a brain-peptide digest. The digest was prepared by incu-

bating brain tissue with the peptide in the presence of ammonia-ammonium chloride buffer and 0.001 M MnCl_2 . The titration was carried out with 0.01 N NaOH after the digestion had been stopped by the addition of formol. The method was one developed for the characterization of a leucyl peptidase in muscle.¹ In the case of this peptidase, MnCl_2 has been shown to have an activating effect (15), and for this reason it was used in all of the brain peptidase studies. A comparison of the peptidase of beef muscle, calf brain, and acetone-ether-extracted brain is shown in Table II.

Autolysis of Brain Tissue—It is evident from Fig. 1 that the rate of autolysis in brain tissue decreases rapidly and that the process is practically at an end in about 48 hours (at 100m temperature). The amount of pro-

TABLE II
Comparison of Brain and Muscle Peptidase Activity

Substrate, 0.05 mM per ml	Per cent hydrolysis,* 20 hrs, † 40°, pH 7.0-7.5		
	Beef muscle, 10 mg per ml	Calf brain, 10 mg per ml	Acetone-ether extracted calf brain, 1.5 mg (= 10 mg fresh tissue per ml)
Glycyl-L-leucine	7 (pH 6.8)	60	22
Glycylglycine	20 (" 6.7)	76 (pH 7.7)	1
dl-Alanylglycine	Complete hydrolysis (17 mg beef muscle)	38	31
dl-Leucylglycylglycine	" "	Complete hydrolysis (pH 8.2)	Complete hydrolysis (1 mg extracted brain)
dl-Leucylglycine†	9 (pH 7.8)	12 (pH 7.7)	14 (pH 7.8)

* Calculated on the basis of the splitting of one peptide bond per molecule

† The figures for dl-leucylglycine are for 2 hour digestion periods

tein breakdown as indicated by the liberation of tyrosine is not considerable and corresponds very closely to the amount liberated in muscle tissue under similar conditions, despite the fact that brain tissue appears to contain much more proteinase than muscle.

In one experiment the trichloroacetic acid filtrates were also analyzed for free amino groups by the Van Slyke procedure (16). The results are shown in Fig. 2. Although these figures indicate a decrease in the autolytic rate between the 3rd and the 5th days, Gibson, Umbreit, and Bradley (3) showed that the free amino acids continued to increase even up to 30 days at the more acid pH. While peptidases are known to be present in brain tissue, there is no clear evidence that the splitting of peptides played

¹ Balls, A. K., and Schwimmer, S., unpublished data.

an important part in the process of autolysis for the first few days, since they are more active at an alkaline pH and the curves in Fig. 2 indicate much greater autolysis at pH 3.5 than at 6.6.

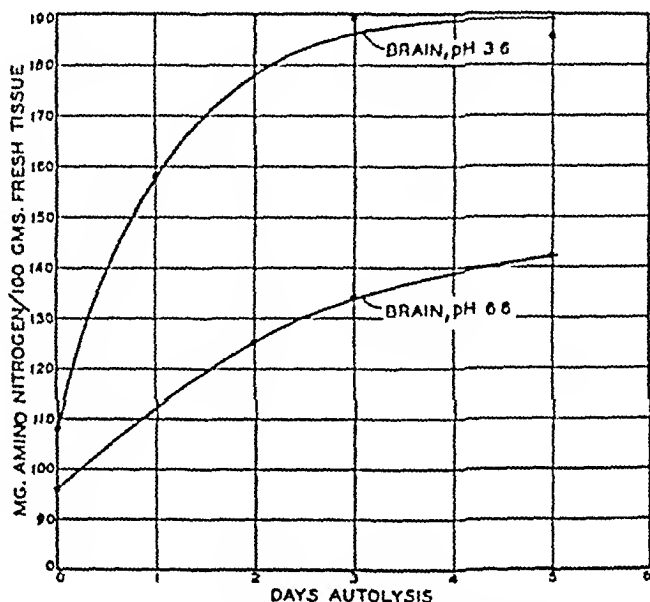


FIG. 2. Increase in free amino groups during autolysis in brain tissue

TABLE III
Survival of Cathepsin in Autolyzing Brain Suspensions

pH	Time	Cathepsin units per gm. tissue $\times 10^4$
6.7	0 hr.	8.5
	24 hrs.	6.3
	48 "	6.3
	72 "	6.4
3.5	0 hr.	8.7
	24 hrs.	4.3
	48 "	5.0
	72 "	5.5
	120 "	5.0
	12 days	4.3

The cessation of autolysis in brain is not due to the destruction of the enzyme. Table III shows the amount of cathepsin surviving during autolysis conducted over several days. While some decrease in the en-

zyme was noted, there was still considerable left even after 12 days at pH 3.5. The addition of purified brain cathepsin to an already completely autolyzed suspension produced no further increment of non-precipitable tyrosine. It was thus impossible to distinguish between the alternatives that the enzyme is inhibited by its end-products or has split the protein as far as it can. The probability of the latter explanation is greater, because if the enzyme were inhibited by end-products the addition of considerable new enzyme would be likely to overcome the inhibition, at least to some extent. The experiments, however, showed no additional digestion whatever.

SUMMARY

Brain proteinase has been partially purified and shown to be of the catheptic type. Di- and tripeptidases were found to be present in brain tissue. No evidence was obtained for the presence of a lipolytic enzyme similar to the one previously reported in muscle.

The results indicate the presence of a surprisingly large amount of cathepsin in brain as compared with muscle. Brain, however, autolyzes neither more rapidly nor to a greater extent than muscle under similar conditions. While the effect of the brain cathepsin undoubtedly contributes to the difficulty of handling brain tissue commercially, it does not seem to afford adequate explanation of the rapid disintegration of the material that is so frequently observed in the packing industry.

BIBLIOGRAPHY

1. Levene, P. A., and Stookey, L. B., *J. Med. Research*, **10**, 212 (1903).
2. Craifaleanu, A. D., *Boll. Soc. nat. Napoli*, **30**, 173 (1917).
3. Gibson, C. A., Umbreit, F., and Bradley, H. C., *J. Biol. Chem.*, **47**, 333 (1921).
4. Gueorgievskaia, A. M., *Russ. Physiol. J.*, **4**, 53 (1922).
5. Takasaka, T., *Biochem. Z.*, **184**, 390 (1927).
6. Slowtsoff, B. J., *Russ. Physiol. J.*, **3**, 1 (1921).
7. Edlbacher, S., Goldschmidt, E., and Schlappi, V., *Z. physiol. Chem.*, **227**, 118 (1934).
8. Blum, E., Jakovtshuk, A. I., and Jarmoschkewitsch, A. I., *Bull. biol. et méd. exp. U. R. S. S.*, **1**, 17 (1936).
9. Hertzman, A. B., and Bradley, H. C., *J. Biol. Chem.*, **61**, 275 (1924).
10. Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
11. Anson, M. L., *J. Gen. Physiol.*, **23**, 695 (1940).
12. Abderhalden, E., and Caesar, G., *Fermentforschung*, **16**, 255 (1940).
13. Matlack, M. B., and Tucker, I. W., *J. Biol. Chem.*, **132**, 663 (1940).
14. Ossovsky, I. A., *Russ. Physiol. J.*, **2**, 68 (1919).
15. Berger, J., and Johnson, M. J., *J. Biol. Chem.*, **130**, 641 (1939).
16. Van Slyke, D. D., *J. Biol. Chem.*, **12**, 275 (1912).

THE WATER-SOLUBLE RIBOFLAVIN-BORON COMPLEX

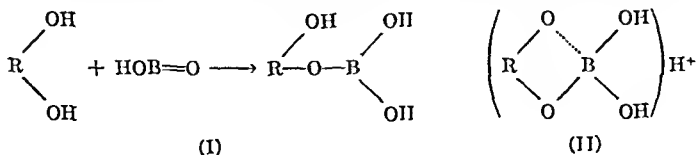
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(Received for publication, August 13, 1942)

In the course of attempts to find water-soluble derivatives of riboflavin, a study was made of the complexes formed between riboflavin and compounds of boron. The discovery was made that when proper pH control is exercised stable solutions of riboflavin-boron complex can be prepared of at least 25 times the natural solubility of riboflavin; i.e., a concentration of about 0.3 per cent as against 0.012 per cent. The nature of the riboflavin-boron complex has been characterized in part; however, all phases of the solvent effect are not clear. A brief consideration of the knowledge of organoboron complexes will serve as an introduction to the problem.

Boric acid has long been known to form complexes and esters with polyhydroxy compounds (1). Both aliphatic and phenolic compounds have received considerable attention and recently Scudi, Bastedo, and Webb (2) described a complex in which both phenolic and aliphatic groups of pyridoxine are bound by boric acid. Brigl and Grüner (3) and Vargha (4) have described the preparation under anhydrous conditions of glucosoboric acid esters. Such esters, however, are unstable in water. Brigl and Grüner state that the mechanism of the boric acid effect with polyhydroxy compounds is not entirely clear but probably occurs in two phases. They picture the first phase of the reaction as that shown in (I).



The boron atom has been shown in many instances to act as having 4 coordinate valences and involvement of two hydroxyl groups is pictured in (II). This formulation serves to explain the accentuation of the acidity of one hydroxyl group and accounts for the strong monobasicity of boric acid in such complexes.

Kuhn and Weygand (5) had shown that boric acid catalyzes the condensation of *o*-diamines with alloxan. This reaction is useful in the synthesis of riboflavin but was shown to be independent of action on the sugar side chain and has not been explained. On the other hand the effect of

borax, described by Kuhn and Rudy (6), to reverse the optical rotation of riboflavin in alkaline solution clearly involves the sugar group.

The following experiments were conducted to perfect a method of preparing stable soluble preparations of riboflavin and to determine the nature of the riboflavin-boron complexes involved.

EXPERIMENTAL

Addition of a small amount of orthoboric acid, metaboric acid, or borax was found to increase the speed of solution of riboflavin in water on heating. Such solutions, in which heating was stopped as soon as all of the riboflavin was dissolved, were found to be physically unstable, however, and attempts were made to determine conditions necessary for permanent stability. The importance of pH control was attested by large numbers of experiments with varying ratios of riboflavin and orthoboric acid. When heating was at a minimum just sufficient to bring about complete solution of the riboflavin, the subsequent physical stability on cooling was found to be closely dependent on pH. Thus solutions of 0.05 per cent riboflavin and 0.1 per cent boric acid were found to be stable at pH more alkaline than 7 but were unstable at pH more acid than 7. The interesting discovery was then made that duration of heating influenced physical stability of such solutions. Solutions of the above composition buffered to pH 6.5 with disodium phosphate proved to be quite stable after heating for 3 hours. Chemical and microbiological assays for riboflavin indicated that about 5 per cent of the total riboflavin was destroyed by this protracted heating. Destruction of riboflavin at pH 7 occurred at a rate about double that at pH 6.5. No differences were discernible between the stable heated solutions and the unstable unheated solutions by the following measurements: freezing point depression, electrometric titration, precipitation with heavy metals, and specific rotation. Also, no difference could be detected between the freezing point depression of heated riboflavin-boron complex solution and an equivalent boric acid solution containing no riboflavin. Transmission curves were drawn for solutions of pure riboflavin and riboflavin-boron complex. There was no difference between the two curves.

The time of heating required to produce stable solutions of various concentrations of riboflavin and orthoboric acid at pH 6.5 was found to be about 3 hours at 95°. The importance of extended heating was demonstrated by removing samples from the water bath at regular intervals up to 3 hours and setting them aside for stability. The time of appearance of first crystals ran about as follows: 12 hours for 30 minutes heating, 7 days for 90 minutes heating, and 20 days for 150 minutes heating. Crystallization continued in all of these until equilibrium with the solution was

reached. In time, about the same amount of riboflavin had separated from all of them. 3 hours heating, on the other hand, produced solutions which have failed to crystallize in a period of 12 months. An apparent state of physicochemical stability was reached with 3 hours heating that was not attained with shorter heating. The time of heating needed to produce stable solutions with metaboric acid was less than that with orthoboric acid. Solutions of good physical stability were also obtained by dissolving the riboflavin and boron compound at alkaline pH, heating gently, and adjusting to pH 6.5.

The addition of acid to riboflavin-borate solutions generally caused precipitation of the riboflavin in a short time; however, certain isotonic solutions of low concentration, *i.e.* 1 mg. per cc., have appeared stable for many months following addition of acid. Solutions containing 3 mg. per cc. of riboflavin stabilized with 5 per cent of boric acid invariably underwent rapid crystallization when small additions of acid were made.

A clear cut stoichiometric relationship between the molecular ratios of riboflavin and boric acid which produced stable solutions on heating was not apparent in our experiments. The requirement for boric acid increased markedly with higher levels of riboflavin. The lowest level of boric acid studied was 0.1 per cent and this was used to dissolve 0.05 per cent riboflavin, a molecular ratio of boric acid to riboflavin of 1:12. The maximum solubility of boric acid in water at ordinary temperatures is about 5 per cent. In the pH range of 6.4 to 6.6, this amount of boric acid was found to hold in solution about 0.3 per cent riboflavin, a molecular ratio of about 1:100.

Addition of riboflavin to boric acid or borax solution caused a very slight lowering of the pH, but no difference was noted in the general appearance of the curves for electrometric titration of boric acid solution in presence or absence of riboflavin. When monohydroxy and polyhydroxy alcohols, such as ethyl alcohol and glycerol, are used to enhance the monobasicity of boric acid, large excesses of the alcohol are used to obtain maximum effect. Such a procedure was not possible in this study because of the limited solubility of riboflavin.

Optical Rotation of Riboflavin-Boron Complex—The ability of compounds of boron to reverse the optical rotation of riboflavin was found to be a function of the pH of the solution and bears an apparent close relationship to the solvent effect. The rotation of riboflavin below pH 6 is enhanced in a negative direction by boron; above pH 6, it is enhanced in a positive direction. Likewise, the solvent effect of compounds of boron is small below pH 6 and increases above pH 6.

The increase in positive rotation of riboflavin-boron solutions with increase in pH is in direct contrast to the increase in negative rotation of

solutions of riboflavin alone with similar increase in pII. The effect of pH on the specific rotation of riboflavin and riboflavin-boric acid solutions is shown in Table I.

The interesting theoretical observation was made that *d*-araboflavin¹ dissolved by boric acid also shows reversal of the sign of rotation as well as accentuation of the degree of rotation on either side of pII 6 (Table II). It seems likely that this reversal of the sign and accentuation of the degree of rotation will apply for other similar sugar derivatives.

TABLE I

Effect of pH and Addition of Boric Acid on Optical Rotation of d-Riboflavin

<i>d</i> -Riboflavin, 0.1 per cent		<i>d</i> -Riboflavin, 0.1 per cent, + boric acid, 1.4 per cent	
pH	$[\alpha]_D^{25}$	pH	$[\alpha]_D^{25}$
	degrees		degrees
1	± 2	1.3	-33
6	± 2	3.0	-30
7.9	-33	6.3	+50
9.8	-55	6.6	+100
11	-78	9.2	+278
12	-89	11.8	+340

TABLE II

Effect of pH and Addition of Boric Acid on Optical Rotation of d-Araboflavin

<i>d</i> -Araboflavin, 0.1 per cent		<i>d</i> -Araboflavin, 0.1 per cent, + boric acid, 1.4 per cent	
pH	$[\alpha]_D^{25}$	pH	$[\alpha]_D^{25}$
	degrees		degrees
3	0	3.3	+10
6.3	+10	6.6	-80
8.5	+20	9.2	-374
11.2	+30	11.6	-410

Further evidence that the ribityl group is involved in the solvent reaction with boric acid and that the effect is largely independent of the very insoluble isoalloxazine group was obtained in the following experiments. Lumiflavin, 6,7,9-trimethylisoalloxazine, was made according to the method of Warburg and Christian (7). Boric acid did not enhance the solubility of this compound or of tetraacetylriboflavin.² Thus, when the sugar group is either absent from the flavin molecule or is completely substituted, no reaction with boric acid appears to occur.

¹ Kindly furnished by Dr. H. E. Zaugg.

² Kindly furnished by Dr. D. M. Hegsted.

Evidence that the isoalloxazine grouping of riboflavin is not involved in complex formation with compounds of boron was obtained indirectly as follows: Reduction of riboflavin-boron complex with sodium hydrosulfite paralleled that of free riboflavin. The simple reversible air oxidation of the colorless dihydroisoalloxazine derivative thus obtained was accomplished with equal rapidity in each case. Photolysis of solutions of free riboflavin and riboflavin-boron complex yielded lumiflavin at about an equal rate. Equal precipitation of the two forms with salts of silver, copper, mercury, and lead indicated that the slightly acidic imide grouping is not blocked by boron.

Preparation of Tetrabenzoylriboflavin—Attempts were made to benzoylate riboflavin in the presence of borates according to the Schotten-Baumann method. No reaction took place, indicating that the hydroxyl groups were completely blocked.

Tetrabenzoylriboflavin was prepared in the following way from free riboflavin. 100 mg. of riboflavin were dissolved in 100 cc. of cold water with 0.6 gm. of sodium hydroxide. 1 gm. of benzoyl chloride was added and the cold solution was extracted four times with ethyl ether. The red crystalline mass which had separated during the benzoylation went slowly into solution in the ether. The combined ether extracts were freed from ether and the residue was dissolved in hot alcohol. On cooling, orange-red crystals were obtained which were extremely insoluble in water and rather sparingly soluble in acetone, ether, and alcohol; m.p. 131–136° (hot stage).

Analysis—Riboflavin tetrabenzoate, $C_{45}N_4H_{16}O_{10}$

Theory. C 68.2, H 4.65, N 7.03

Found. " 68.7 " 4.72 " 7.00

Preparation of Boric Acid Esters—Concentration *in vacuo* of the soluble riboflavin-boron complex has invariably resulted in the appearance of inseparable free riboflavin and boric acid crystals. However, when riboflavin was heated for an hour with metaboric acid (molecular ratio 1:2) in glacial acetic acid, homogeneous crystals in good yield were obtained on cooling; m.p. 290–292° (uncorrected). The analysis of the crystals fitted those of riboflavin monoborate.

Riboflavin-monoborate, $C_{17}H_{11}O_4N_4B$, mol. wt. 419

Theory. C 48.5, H 5.01, N 13.88, ash (B_2O_3) 8.3, B 2.58

Found. " 47.2, " 5.13, " 13.34, " " 8.01, " 2.58

Riboflavin (fluorescence analysis). Theory, 89.4; found, 89.4

The specific rotation of the compound was +62.5° at pH 7.7 and +120° at pH 11. Addition of further boric acid at pH 11 caused an increase in rotation to +275°. This compound dissolved readily with shaking in water in a concentration of 0.05 to 0.1 per cent, but apparently underwent

rapid hydrolysis, since crystals appeared soon after solution was complete and crystallization progressed rapidly until most of the riboflavin had separated from solution.

When a molecular excess of metaboric acid of 4:1 was used in glacial acetic acid in an attempt to produce the diborate, homogeneous crystals were evidently not obtained. The material dissolved readily in water and required somewhat longer for precipitation of free riboflavin than did the monoborate described above. The melting point (uncorrected) was 290-292°, but the fluorometric analysis indicated the presence of about 84 per cent riboflavin as against 89.7 per cent for the monoborate and 81 per cent calculated for the diborate. When metaboric acid and riboflavin (4:1) were heated together in acetone with a small amount of pyridine to complete the solution, a crystalline product separated on cooling which had 93 per cent riboflavin by analysis. This product dissolved in part rapidly in water, but almost immediately hydrolyzed and precipitated free riboflavin. Thus material was obtained in which only part of the riboflavin was esterified and other material in which the esterification was probably complete but involved only part of the eligible groupings. The position of the boron substituent as regards the four hydroxyl groups is problematical. The marked effect on specific rotation shown by the riboflavin-monoborate suggests that the carbon atom 2 is involved according to the theory of Weygand (8).

DISCUSSION

Although the capacity of derivatives of boron to dissolve riboflavin in water appears to be clearly concerned with complex formation with the hydroxyl groups of the ribityl side chain, the nature of the complex is not clear. The facts at hand suggest that the soluble complex exists in at least two forms, one of which, produced by long heating, is relatively stable, and the other, produced by short heating, is relatively unstable. The fact that synthetic riboflavin monoborate undergoes rapid hydrolysis with separation of riboflavin from solution suggests that the ester type of linkage of Brigl and Grüner (3), previously discussed, may form to some extent but is not equivalent to the condition which occurs in the stabilized aqueous solutions. Rather large molecular excesses of boron compound are needed to produce stable solutions, suggesting a physicochemical relationship involving an equilibrium between a soluble ester form and free insoluble riboflavin. The slight increased acidity of riboflavin-boron solutions and the close dependence of solubility on pH add further evidence to the presence of a highly dissociated ester type of linkage in which the monobasicity of boric acid is enhanced in the well known manner. The action of heat to produce stable complex formation cannot be explained satisfactorily,

since no physical or chemical differences can be detected between the heated and unheated solutions.

Weygand (8) has concluded from extensive studies on the rotation of flavin glycosides that in general more than three-fourths of the degree of rotation of this type of compound is due to the grouping on carbon atom 2 of the polyoxyalkyl side chain. The great effect of the addition of boron compounds on the direction and degree of rotation of *d*-riboflavin and *d*-araboflavin points to involvement of carbon atom 2 in the sugar side chain. Involvement of all four hydroxyl groups in aqueous solution is indicated also by complete success of benzylation in absence of boric acid and complete failure in its presence. Full biological activity of the stable riboflavin-boron complexes formed in aqueous solution with heating is indicated both by the activity of all preparations in the microbiological assay and by separate animal experiments. In assays with rats, riboflavin-boric acid complex administered by intramuscular injection three times weekly was as effective in promoting growth as equivalent amounts of riboflavin in water by mouth. The material has also been used successfully by injection to supply the riboflavin requirement of dogs on synthetic rations over periods of several months. The amount of boric acid obtained in the injection fluid is negligible and is excreted with extreme rapidity (9-12).

The solvent effect of boron compounds is quantitatively more than 15 times as great as that reported by Schou and Fretheim (13) for urea and urethane and is about 3 times as effective as nicotinamide in low concentrations as determined in our laboratories. Kuhn (14) reported the use of sodium desoxycholic acid and *N*-methylacetamide as solvent agents for riboflavin, but neither compound has proved suitable in our hands.

Isotonic preparations of the riboflavin-boron complex containing 1 mg. per cc. of riboflavin have been shown to be self-sterilizing towards molds and bacteria. In the course of nutritional experiments, sterile solutions have been injected several times weekly into rats and dogs for long periods of time with non-sterile syringes and needles. No abscesses or other signs of infection at the point of injection have appeared as commonly occurs when non-sterile techniques are used. Similar solutions have been tested for acute and chronic toxicity in rats and dogs. No deleterious effects were seen as affecting growth, estrus, the blood picture, or the general well being of the animals.

SUMMARY

1. Preparations of soluble riboflavin-boron complexes containing up to 0.3 per cent riboflavin in solution are described. The preparations have proved suitable for injection.

2. Evidence for the nature, in part, of the soluble complex has been obtained.

3. Synthesis of riboflavin monoborate and tetrabenzoylriboflavin are described.

4. The effect of pH on the specific rotation of *d*-riboflavin, *l*-araboflavin, and boron complexes formed by them has been studied.

Thanks are expressed to Eleanor Willerton, F. Peirce Dann, and Elmer O. Krueger for the microbiological, animal, and chemical riboflavin assays run during the course of this study. Thanks are also expressed to Mr. Carl Nielsen and Dr. A. E. Knauf for helpful consultation throughout and to Dr. H. W. Cromwell and Dr. R. K. Richards for the bacteriological and pharmacological studies reported.

BIBLIOGRAPHY

1. Boeseken, J., *Rec. trav. chim. Pays-Bas*, **40**, 553, 568, 578 (1921); *Ber. chem. Ges.*, **46**, 2612 (1913).
2. Scudi, J. V., Bastedo, W. A., and Webb, T. J., *J. Biol. Chem.*, **136**, 399 (1940).
3. Brigl, P., and Grüner, H., *Ann. Chem.*, 495, 60 (1932); *Ber. chem. Ges.*, **66**, 1977 (1933); **67**, 1969 (1934).
4. Vargha, L., *Ber. chem. Ges.*, **66**, 704, 1394 (1933).
5. Kuhn, R., and Weygand, F., *Ber. chem. Ges.*, **68**, 1282 (1935).
6. Kuhn, R., and Rudy, H., *Ber. chem. Ges.*, **68**, 169 (1935).
7. Warburg, O., and Christian, W., *Biochem. Z.*, **266**, 377 (1933).
8. Weygand, F., *Ber. chem. Ges.*, **73**, 1271 (1940).
9. Kahlenberg, L., and Barwasser, N., *J. Biol. Chem.*, **79**, 405 (1928).
10. Hove, E., *Am. J. Physiol.*, **127**, 689 (1939).
11. Kent, N. L., and McCance, R. A., *Biochem. J.*, **35**, 812 (1941).
12. Peyton, H. A., and Green, D., *South. Med. J.*, **34**, 1286 (1941).
13. Schou, S. A., and Fretheim, B., *Dansk Tidsskr. Farm.*, **14**, 97 (1910); *Pharm. Abstr.*, *J. Am. Pharm. Assn.*, **6**, 503 (1940).
14. Kuhn, R., *Klin. Woch.*, **17**, 222 (1938).

MOLECULAR KINETIC AND ELECTROPHORETIC STUDIES ON CARBONIC ANHYDRASE

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The purified carbonic anhydrase prepared from ox blood by Scott and Fisher (3) appeared to be almost a pure substance. They reported, "Adsorption, electrodialysis, or fractionation with inorganic salts and solvents did not result in any marked increase in the activity of this product." The amorphous powder contained about 0.2 per cent zinc and had a nitrogen content of 15.8 per cent. Its activity was approximately 10,000 units per mg. of solids. The activity of the crystals was about 9000 units.

In 1940 Keilin and Mann (2) described a preparation of carbonic anhydrase which they believed to be almost pure. Enzyme prepared by two different methods and from two sources—red blood cells and gastric mucosa—had about the same activity, which could not be increased; and the preparation showed only one component on electrophoresis. This material contained 14.9 per cent nitrogen and 0.33 per cent zinc. Its activity was 2220 units per mg.

The difference in activity reported for these preparations by the two groups of workers may be related to a difference in chemical composition or may be due to the fact that Scott and Fisher used a stabilizer in their solutions during testing. In any event, constant activity seems inadequate as a sole criterion of purity. The sedimentation behavior, diffusion rate, and electrophoretic mobility of the carbonic anhydrase prepared by Scott and Fisher have accordingly been studied in this laboratory.

Methods

The preparations of carbonic anhydrase studied have been described in detail by Scott and Fisher (3). Sample CA-1 corresponds to the material in their Experiment 1. It was a purified amorphous preparation which had been dialyzed and brought from Toronto to Madison at room temperature. Sample CA-3 was similar to Sample CA-1 except that the dialysis and transportation to Madison were accomplished in the cold. Sample CA-2 had been crystallized from another portion of Sample CA-1 as described in Experiment 3 of Scott and Fisher and dried *in vacuo*. It was taken up in water and the small amount of insoluble material present removed by centrifugation. All three samples were dialyzed in the cold against phosphate-borate buffer, pH 7.1, containing 1 per cent sodium

chloride, for sedimentation and diffusion experiments, or against the buffers used for electrophoresis. Sample CA-3 was also centrifuged in water solution. Sample CA-4 was prepared by treatment with basic lead acetate (4) and the excess lead removed as the phosphate. The final solution had an enzyme concentration of approximately 2 per cent and contained about 1 per cent mixed phosphate.

The sedimentation behavior of carbonic anhydrase was studied in the Svedberg oil-driven ultracentrifuge at 60,000 r.p.m. The position of the boundaries was observed by the Lamm scale method. Sedimentation constants were calculated in the usual manner (5). The areas under the line displacement-distance diagrams were measured with a planimeter, and the relative amounts of protein represented by each peak calculated. In order to obtain the maximum resolving power, the cell was filled with solution; so that no correction for distance from the meniscus could be made.

The diffusion constant measurements were made in a Lamm cell. The blurring of the boundary was followed by the scale method, and the diffusion constant calculated by the method of moments.

The electrophoretic mobility of the enzyme was studied in buffers of ionic strength 0.10 at pH 5.0, 6.0, 7.0, and 9.0 in the Tiselius electrophoresis apparatus. The positions of the boundaries were observed by the Svensson-Philpot schlieren method.

Results

These preparations were almost homogeneous in the ultracentrifuge. The line displacement-distance diagram obtained on Sample CA-3 after 87 minutes at 60,000 r.p.m. is shown in Fig. 1. The curve shows a slight asymmetry; evidently some material is present which sediments more rapidly than the chief component. Every sample studied showed the same type of curve. The results of the sedimentation experiments are shown in Table I. Enzyme studied before dialysis (Sample CA-4) or after crystallization (Sample CA-2) contained the same amount of heavy material. Neither the nature of the solvent (Samples CA-3, a and b) nor the protein concentration during centrifuging seemed to affect the relative proportion of heavy protein present.

The sedimentation constant of carbonic anhydrase is 2.8 S. (svedbergs),¹ and the rate of sedimentation is independent of the protein concentration up to 1 per cent protein. This value is close to that obtained by Philpot, 3.0 S. (1).

The results of three diffusion experiments are also shown in Table I. The best average value for the diffusion constant appears to be 9.0×10^{-7}

¹ 1 svedberg = 10^{-13} cm. per second per unit of force.

sq. cm. per second. Although the individual values fall off somewhat with time, the line displacement-distance diagrams obtained are very

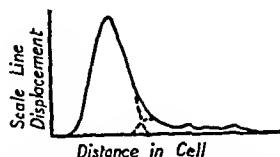


FIG. 1. Carbonic anhydrase (Sample CA-3) after 87 minutes centrifuging at 60,000 R.P.M. The broken lines indicate the separate components.

TABLE I
Carbonic Anhydrase

Sample No	Protein concentration	Solvent	Per cent in main peak	s_{20}
Sedimentation				
CA-1	1.0	Phosphate-borate, pH 7.1, 1% NaCl	79	S. 2.8
	0.5	" " 7.1, 1% "	85	2.8
CA-2	1.0	" " 7.1, 1% "	86	2.7
CA-3a	1.0	" " 7.1, 1% "	85	2.8
b	1.0	Water	90	
CA-4	2.0	Phosphate	83	
Diffusion				
CA-1	0.5	Phosphate-borate, pH 7.1, 1% NaCl	Time	D_{20}
			sec.	$\frac{\text{cm.}^2 \text{ per sec} \times 10^7}{\text{sec}}$
			42,750	9.3
			52,740	9.3
CA-2	1.0	" " 7.1, 1% "	66,900	8.2
			82,320	8.7
			61,440	10.6
	1.0	" " 7.1, 1% "	72,360	10.7
			83,040	9.8
			33,540	9.4
			74,760	8.8
			82,080	8.7

close to ideal distribution curves. The diffusion constants obtained are therefore probably close to the true value for the pure enzyme.

The partial specific volume of this material has not been measured. If

0.749, the value for insulin crystals containing zinc, is used (5), the molecular weight is calculated by the formula (5),

$$M = \frac{RTs}{D(1 - v\rho)}$$

to be approximately 30,000.

The three preparations studied (Samples CA-1, 2, and 3) behaved similarly on electrophoresis in phosphate buffer at pH 7.0. Each showed one large peak and about 10 per cent of slightly faster material (Fig. 2). The peaks all show spreading with time, which continues after reversal of the current and so is ascribable to the rapid diffusion rate of the material rather than to any electrical inhomogeneity. Sample CA-3 was also studied in phosphate buffer at pH 6.0 and in acetate at pH 5.0. Sample

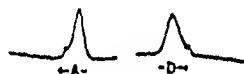


FIG. 2

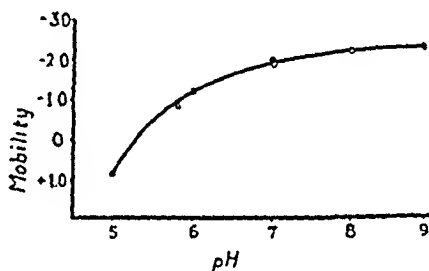


FIG. 3

FIG. 2. Electrophoresis of carbonic anhydrase (Sample CA-2) at pH 7.0.

FIG. 3. Electrophoretic mobility of carbonic anhydrase calculated from the descending boundaries. Mobilities are given in sq. cm. per second per volt $\times 10^5$. ● represents the authors' data; ○ represents the data of Keilin and Mann.

CA-1 was studied at pH 9.0 in veronal buffer. The pH-mobility curve obtained is shown in Fig. 3. The mobilities obtained by Keilin and Mann (2) have been plotted for comparison; the agreement is very good. The isoelectric point appears to be at pH 5.3 in solutions of ionic strength 0.10. At pH 5.0 the small peak is slightly slower than the main peak.

DISCUSSION

These preparations of carbonic anhydrase have been purified by chemical means until a state of constant activity has been reached. Nevertheless, sedimentation and electrophoresis studies reveal about 15 per cent of impurity. This impurity sediments slightly faster than the enzyme and seems to have a somewhat lower isoelectric point. Electrophoretic purification of the enzyme would be difficult because of the similar mobilities of the enzyme and the impurity and their high diffusion rates.

SUMMARY

1. Carbonic anhydrase has a sedimentation constant of 2.8 S., a diffusion constant of 9.0×10^{-7} sq. cm. per second, and a molecular weight of 30,000.

2. The electrophoretic mobility of the enzyme has been determined over the pH range of 5.0 to 9.0 at constant ionic strength. Its isoelectric point is at pH 5.3.

3. Both amorphous and crystalline preparations, with activities of 9000 or more units per mg. of solids, contain about 15 per cent of impurity.

This study was undertaken at the request of Dr. D. A. Scott and Dr. A. M. Fisher, who furnished all the enzyme preparations used.

The authors wish to express sincere thanks to J. W. Williams for advice and encouragement in this work. The expenses of this investigation were defrayed by the Wisconsin Alumni Research Foundation.

BIBLIOGRAPHY

1. Eirich, F. R., and Rideal, E. K., *Nature*, **146**, 541 (1940).
2. Keilin, D., and Mann, T., *Biochem. J.*, **34**, 1163 (1940).
3. Scott, D. A., and Fisher, A. M., *J. Biol. Chem.*, **144**, 371 (1942).
4. Scott, D. A., and Mendive, J. R., *J. Biol. Chem.*, **139**, 661 (1941).
5. Svedberg, T., and Pedersen, K. O., *The ultracentrifuge*, Oxford (1940).

LETTERS TO THE EDITORS

THE USE OF MYLASE P IN THE PREPARATION OF NATURAL MATERIALS FOR MICROBIOLOGICAL PANTOTHENIC ACID ASSAY

Sirs:

As soon as the microbiological pantothenic acid assay of Pennington, Snell, and Williams¹ was published, we tried it for the assay of several natural materials. It was immediately apparent that the resulting values in most cases were only a fraction of those obtained by the chick assay of Jukes² as used in our laboratory.

Pennington *et al.*¹ suggested autoclaving natural materials, or autolysis under benzene prior to autoclaving, to free additional pantothenic acid. In our experience such treatments also failed to yield anticipated values. Since enzymatic hydrolysis was already being used to free combined forms of certain B vitamins, we were prompted to try several commercial enzymes in the preparation of the sample for assay. Of those tried, mylase P³ was the most satisfactory.

Recently other enzymatic methods^{4,5} for the preparation of samples for pantothenic acid assay have been published. From our use of mylase for over 1½ years we feel that it offers certain advantages.

Weigh 0.4 gm. of sample and 0.4 gm. of mylase P³ into a 50 cc. tube. Add 10 cc. of 2 per cent acetic acid and 1 cc. of 1 N NaOH; mix thoroughly (pH approximately 4.2) and incubate at 50° for 2 hours with occasional stirring, or at 37° overnight. The contents of the tubes are then diluted to about 30 cc. and the pH adjusted to 5 to 6 by the addition of approximately 1.5 cc. of 1 N NaOH. The mixture may be used immediately at this point or stored in the refrigerator for use the next morning. The material is transferred to a volumetric flask and suitable dilutions made for assay.

As in the case of clarase,⁶ each lot of mylase has been found to have a

¹ Pennington, D., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **135**, 213 (1940).

² Jukes, T. H., *J. Biol. Chem.*, **117**, 11 (1937).

³ Mylase P (special) for thiamine determination, obtained from Wallerstein Laboratories, 180 Madison Avenue, New York. Each lot of mylase has had a different activity; e.g., with one lot, maximum results were obtained by using 0.1 gm. of mylase to 0.4 gm. of sample. The activity of each new lot was determined.

⁴ Waisman, H. A., Henderson, L. M., McIntire, J. M., and Elvehjem, C. A., *J. Nutrition*, **23**, 239 (1942).

⁵ Strong, F. M., Feeney, R. E., and Earle, A., *Ind. and Eng. Chem., Anal. Ed.*, **13**, 566 (1941).

⁶ Willerton, E., and Cromwell, H. W., *Ind. and Eng. Chem., Anal. Ed.*, **14**, 603 (1942).

different content of pantothenic acid which must be taken into account. In the assay of some materials of low potency with a particular lot of mylase when the blank was higher than desirable, 75 per cent of the pantothenic acid was removed from the enzyme preparation by dialysis at 5° in a collodion bag for 12 hours without lowering the enzyme activity.

Comparative Assay Results of Several Materials after Enzymatic Digestion

Test material	Ca pantothenate per gm.	
	Mylase digested* 2 hrs.	Clarase digested† 48 hrs.
	γ	γ
Brewers' yeast.....	83.7	79.8
" "	78.2	74.9
" "	103	109
" "	195	206
Dried yeast extract.....	539	544
" " "	304	249
Liver concentrate powder	388	377

* 0.4 gm. of enzyme to 0.4 gm. of sample.

† 1.0 gm. of enzyme to 0.5 gm. of sample.

The table shows comparative assay results on several materials after treatment for 2 hours with mylase and 48 hours with clarase.⁵ In both instances the pantothenic acid content of the digests was determined by the procedure of Strong, Feeney, and Earle.⁵

The results by the two methods are in excellent agreement. The obvious advantage of the mylase method is the shorter digestion time plus the fact that a considerably smaller quantity of enzyme is required.

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ISOLATION OF DIHYDROSPHINGOSINE FROM BRAIN AND SPINAL CORD*

Sirs:

In the recrystallization of crude sphingosine sulfate prepared by various methods from brain and spinal cord, we have consistently obtained a fraction which is only slightly soluble in methyl alcohol. Acetylation of this material yielded a considerable proportion of triacetyldihydrosphingosine. The presence of dihydrosphingosine in nervous tissue has not been reported previously, although Lesuk and Anderson¹ obtained the saturated base from the cerebrosides of *Cysticercus fasciolaris*. This observation, therefore, opens up some interesting problems concerning the metabolism of sphingosine.

The sulfate fractions were acetylated as follows: 5 gm. were shaken in a separatory funnel with 100 cc. of 0.5 N sodium hydroxide, 200 cc. of ether, and 20 cc. of methyl alcohol until no solid particles remained. Then 3.0 gm. of acetic anhydride and 30 cc. of 2.5 N sodium hydroxide were added and the mixture was shaken vigorously until the odor of acetic anhydride disappeared. Occasionally a solid separated at this point, necessitating the addition of more methyl alcohol. The ether layer was separated, dried, and concentrated to dryness. The residue was dissolved in 200 cc. of methyl alcohol and the solution was cooled in an ice bath, precipitating 2.5 gm. of solid. This material (presumably the N-acetyl derivative) was further acetylated by the method of Klenk and Diebold.² The product was recrystallized twice from ethyl alcohol, giving 2.2 gm. of crystalline solid, m.p. 98–100°; specific rotation $[\alpha]_D^{30} = +18.0^\circ$. The rotations were determined on a 1 per cent solution in chloroform (0.1 gm. of triacetyldihydrosphingosine per 10 cc. of chloroform). Analysis, C 67.20, H 10.60, N 3.41; calculated for $C_{24}H_{45}O_6N$, C 67.45, H 10.54, N 3.28. The melting point and specific rotation are identical with those of an authentic sample of triacetyldihydrosphingosine, which was prepared by reducing sphingosine to dihydrosphingosine and acetylating the latter in pyridine.³

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* The authors wish to thank The Upjohn Company for a Research Fellowship in support of this work.

¹ Lesuk, A., and Anderson, R. J., *J. Biol. Chem.*, **139**, 457 (1941).

² Klenk, E., and Diebold, W., *Z. physiol. Chem.*, **198**, 25 (1931).

³ Carter, H. E., and coworkers, unpublished data.

A GLUTAMINE-LIKE SUBSTANCE IN BLOOD PLASMA

Sirs:

In picric acid filtrates of human and dog plasma the following reactions have been observed which are characteristic of glutamine. When the filtrates, with pH about 2, were heated at 100°, the amino nitrogen determinable by the nitrous acid reaction¹ and the carboxyl nitrogen determinable by the ninhydrin-CO₂ method² decreased, while ammonia was liberated. (The decrease in NH₂ and parallel formation of about half as much NH₃ by glutamine solutions heated at various pH levels have been studied by Vickery, Pucher, Clark, Chibnall, and Westall.³ These changes are presumably due to formation of pyrrolidonecarboxylic acid.) Subsequent hydrolysis with 2 N HCl restored all the carboxyl nitrogen in the filtrates and an equal amount of amino nitrogen. Glutamine solutions under similar conditions were found to show the same reactions at approximately the same velocities. In the plasma filtrates the quantitative extent of the reactions would indicate 5 to 10 mg. of glutamine per 100 cc. of plasma. Experiments to isolate the material responsible for the reactions are in progress.

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¹ Van Slyke, D. D., *J. Biol. Chem.*, **83**, 425 (1929).

² Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A., and Hamilton, P., *J. Biol. Chem.*, **141**, 627 (1941).

³ Vickery, H. B., Pucher, G. W., Clark, H. E., Chibnall, A. E., and Westall, R. G., *Biochem. J.*, **29**, 2710 (1935).

THE GROWTH-PROMOTING EFFECT OF FOLIC ACID AND BIOTIN IN RATS FED SUCCINYLSULFATHIAZOLE

Sirs:

In previous experiments¹ it was shown that succinylsulfathiazole (sulfasuxidine²) reduced the growth of rats maintained on a synthetic ration to the same extent as sulfaguanidine. In this note we wish to report the effectiveness of biotin and especially folic acid in counteracting the growth inhibition produced by succinylsulfathiazole.

Weanling rats 18 days old from Sprague-Dawley and our stock colony were used. The basal ration had the following percentage composition: sucrose, 72; casein, 18; corn oil, 5; Salts 4,³ 4; and succinylsulfathiazole, 1. Choline hydrochloride was added to this basal ration at 1 gm. per kilo of ration. The B vitamins were fed daily in supplement dishes at the following levels: thiamine 20 γ , riboflavin 20 γ , pyridoxine 25 γ , and calcium pantothenate 100 γ . 2 drops of haliver oil were given each rat per week.

In a preliminary series, rats were placed on the basal ration and the basal ration with 0.25 and 0.5 gm. of 1:20 liver powder added daily. The lower level prevented loss of weight and the higher level allowed normal growth. Many of our animals maintained on the basal ration and receiving only the synthetic vitamins developed spectacle eye, which indicated a mild biotin deficiency as noted in a previous paper.⁴ The animals would reach a weight plateau or lose weight after 4 to 5 weeks on the basal ration and at this time therapy was started; thus each animal served as a control.

A concentrate of the eluate factor of Snell and Peterson (folic acid) was prepared according to the procedure of Hutchings, Bohonos, and Peterson,⁵ but was purified only to the superfiltrol eluate stage.⁵ Three rats were given 5 mg. of this concentrate (the equivalent of 0.25 gm. of solubilized liver extract) and three rats were given 5 mg. of the concentrate and 1 γ of biotin per day. In all the animals definite growth responses were observed, ranging from 13 to 20 gm. for the 1st week of therapy.

¹ Black, S., Overman, R. S., Elvehjem, C. A., and Link, K. P., *J. Biol. Chem.*, **145**, 137 (1942).

² Sulfasuxidine was kindly supplied by A. D. Welch of Sharp and Dohme, Inc., Glenolden, Pennsylvania.

³ Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, **138**, 460 (1941).

⁴ Nielsen, E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, **48**, 349 (1941).

⁵ Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, **141**, 521 (1941).

⁶ We are indebted to E. B. McQuarrie for this preparation.

The feeding of biotin in addition to the concentrate resulted in better growth responses. However, when biotin was fed or injected alone, the spectacle eye condition improved but the growth responses were very small, due undoubtedly to a limited intake of folic acid.

We obtained from Dr. E. E. Snell of The University of Texas, Austin, a folic acid preparation which was found to be 15,000 times as potent in folic acid as solubilized liver. At present we have obtained growth responses in fifteen rats with various levels of this folic acid preparation. The growth response for the 1st week of therapy ranges from 12 to 31 gm. We have found that 10 γ per day of this preparation are as effective as 100 γ . The therapy has been continued in several animals and in all cases additional increases in weight have been obtained. The deficiency produced by feeding succinylsulfathiazole has been largely overcome by the feeding of folic acid and biotin.

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GLYCOGEN FORMATION FROM PYRUVATE IN VITRO IN THE PRESENCE OF RADIOACTIVE CARBON DIOXIDE

Sirs:

The present communication reports experiments demonstrating (1) glycogen formation, *in vitro*, by rabbit liver slices with pyruvate as substrate, and (2) the degree to which (+4) carbon (*i.e.*, carbon as CO_2 , H_2CO_3 , HCO_3 , CO_3) participates in this synthesis, short lived radioactive carbon, C^{11} , being used for the purpose of marking the (+4) carbon.

The formation of glycogen from pyruvate, *in vitro*, was found to be profoundly influenced by the ionic environment of the nutrient medium, the presence of a high concentration of potassium and a divalent ion, preferably calcium, yielding the best results. The medium giving optimum glycogen formation after 2 hours incubation consisted of CaCl_2 5.6 mm per liter, KCl 75 mm per liter, K pyruvate 60 mm per liter, KHCO_3 43 mm per liter (containing (+4) C^{11}). The incubation was carried out at 38° in 175 cc. flasks containing about 8 gm. of liver slices plus 24 cc. of solution. The gas phase was 95 per cent O_2 :5 per cent CO_2 . The pH of the medium varied from 7.4 to 7.2 during the 2 hour incubation period. The total CO_2 and the radioactivity of the solution were determined at 15, 45, 75, and 120 minute intervals in order to estimate the specific radioactivity of the (+4) carbon during the period of glycogen formation. From these data and from the determinations of the glycogen formed, it was possible to estimate the proportion of glycogen carbon derived from (+4) carbon.

Experiment No.	Glycogen formed	Glycogen carbon	C^{11} in glycogen as per cent of total C^{11} present	(+4) carbon incorporated into glycogen	Glycogen carbon derived from (+4) carbon
	mg.	mm		mm	per cent
1	27.2	0.91	3.32	0.102	11.2
2	16.0	0.53	1.32	0.040	7.5
3	8.0	0.27	1.22	0.045	16.7
4	7.4	0.25	0.80	0.030	12.0
Average.....			1.67		11.9

Four such experiments have been successfully completed and are reported in the table. The results indicate (1) that glycogen may be produced by liver slices *in vitro* from pyruvate as substrate and (2) that approximately 12 per cent of the glycogen so formed has been derived from (+4) carbon. This is the same order of magnitude as has been reported

previously for the incorporation of (+4) carbon in liver glycogen formed by rats *in vivo*.¹

We wish to express our appreciation to the Harvard cyclotron group, and particularly to Dr. B. R. Curtis, for their cooperation, and to the Milton Fund for financial assistance.

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¹ Solomon, A. K., Vennesland, B., Klemperer, F. W., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **140**, 171 (1941). Vennesland, B., Solomon, A. K., Buchanan, J. M., and Hastings, A. B., *J. Biol. Chem.*, **142**, 379 (1942).

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